



Atty Dkt No.2300-1681
Client Dkt No. PP001681.0002
PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:

PALLIARD

Confirmation No.: 3705

Serial No.: 09/894,845

Group Art Unit: 1635

Filing Date: June 27, 2001

Examiner: J. Angell

Title: TOLERANCE AND CHRONIC HEPATITIS C VIRUS

DECLARATION OF MICHAEL HOUGHTON, Ph.D.

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Sir:

I, Michael Houghton, hereby declare as follows:

1. I received my Bachelor's of Science Degree in the Biological Sciences from the University of East Anglia at Norwich, England in 1972, and my Doctor of Philosophy Degree in Biochemistry from King's College, University of London in 1977.

2. I have been employed by Epiphany Biosciences, Inc. since 2007 and currently hold the position of Chief Scientific Officer. I previously served as Vice-President of Hepatitis C and Virology Research at Chiron Corporation and Novartis Vaccines & Diagnostics, Inc., which acquired the technology that is the subject of this application. I am currently a paid consultant for Novartis Vaccines & Diagnostics, Inc. I am extremely familiar with hepatitis C virus (HCV) and, in particular, with HCV pathogenesis, including the development of immune tolerance in infected patients and liver pathology associated with acute and chronic HCV infection, having actively studied and worked in this discipline for over 25 years. I have coauthored numerous publications and patents relating to HCV immunopathology. A copy of my Curriculum Vitae is attached hereto as Exhibit A.

3. I have reviewed relevant documents from the prosecution of the above-referenced application (hereinafter "the application"), including the Office Action dated April 18, 2007 and

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the art cited therein, the Advisory Action dated July 9, 2007, and the claims as amended October 18, 2007. I understand the claims have been rejected over the reference of Gorczynski et al. (Cellular Immunol. (1995) 160:224-231; hereinafter "Gorczynski") in view of Nakai et al. (Blood (1998) 91:4600-4607; hereinafter "Nakai"), and further in view of Wakita et al. (J. Biol. Chem. (1998) 273:9001-9006; hereinafter "Wakita") and Donnelly et al. (WO 97/47358; hereinafter Donnelly).

4. I understand that the claims pending in the application are directed in part to methods of preparing a non-human animal for screening for agents that modulate tolerance to a hepatitis C virus (HCV) immunogen by preparing a nucleic acid directing liver-specific expression of an HCV immunogen, and exogenously delivering the nucleic acid to the liver of the animal by portal vein injection under conditions that result in the sustained expression of the HCV immunogen for at least one month in the liver thereby inducing immunological tolerance to the HCV immunogen.

5. I understand that the claims pending in the application have been rejected in whole or in part based on the teachings of Gorczynski, Nakai, Wakita, and Donnelly. I also understand that the Examiner is of the opinion that it would have been obvious to combine the teachings of these four references, at least in part due to the fact that a person of ordinary skill in the art would have had a reasonable expectation of success in making an animal tolerant to an HCV antigen given that portal vein injection of non-HCV antigens has been used for inducing tolerance to other immunogens.

6. I do not agree the combination of references cited in the Office Action describe the invention claimed or render the claimed invention obvious, i.e., that the differences between the invention as claimed and the subject matter of the cited art are such that they would be obvious to one skilled in the art, such as myself, as of the filing date of the patent application or earlier. My opinion is based on the facts set forth below and my familiarity with the subject matter. In particular, I disagree with the Examiner that one of skill in the art would be motivated to combine the teachings of Gorczynski with those of Nakai, Wakita, and Donnelly, as Gorczynski describes the injection of lymphoid or spleen cells into the portal veins of mice a few

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days before skin graft transplants to delay transplant rejection and fails to describe anything pertaining to HCV.

7. There remains a need in the art to find suitable animal models for screening for agents that can modulate or reverse immunological tolerance to HCV antigens. None of the cited references describe an animal model of immunological tolerance in which antigens are specifically targeted and restricted to the liver. HCV replication occurs almost exclusively in the liver where tolerance to the virus develops due to the specialized liver environment, which limits T cell activation and function. See, e.g., Crispe, IN, *Nat. Rev. Immunol.* (2003) 3:51-62, attached as Exhibit B. The use of liver-specific promoters and enhancers in the animal model of the instant invention restricts expression of HCV antigens to the liver, and therefore more accurately mimics the natural development of tolerance to HCV immunogens in the liver during viral infection.

8. Unlike the animal model of the instant invention, expression of HCV antigens in the transgenic animal model described by Wakita is not liver-specific. Genes encoding HCV antigens are present in every tissue of the transgenic mice and are expressed using a CAG promoter that is not liver-specific (page 9002, col. 1). Furthermore, the adenovirus vector encoding the Cre trans gene, which is used to turn on expression of genes encoding HCV antigens, though injected into the tail vein to target the liver, is not restricted to the liver. In fact, expression of HCV antigens is detected in a variety of tissues outside of the liver in Wakita's transgenic mice, including in the lung, spleen, thymus, kidney, stomach, intestines, and muscles (see page 9004, col. 1). Moreover, in the CN2-29 transgenic mice described by Wakita, the expression of the HCV core antigen is at about the same level in the spleen (5.5 ng/mg) as in the liver (6.6 ng/mg) and only about 2-fold less in the lung (2.9 ng/mg). Thus, the transgenic mice of Wakita express HCV antigens in multiple tissues where immunoreactivity is not dampened in the same way as the liver.

9. Similarly, Gorczynski fails to describe or suggest methods for limiting exposure of antigens to the liver of animals. Gorczynski describes injection of lymphoid or spleen cells into the portal vein of mice; however, the cellular antigens are not restricted to the liver and can

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migrate elsewhere. Thus, both Gorczynski and Wakita describe animal models in which antigens are exposed to the host's immune system outside of the tolerogenic environment of the liver. The instant invention, in contrast, uses liver-specific promoters and enhancers (e.g., alpha-1 anti-trypsin (AAT) promoter and apolipoprotein E (ApoE) enhancer) to ensure that expression of HCV antigens is restricted to the liver, better mimicking the natural biology of the virus and the evolution of tolerance.

10. The Wakita transgenic animal model also has a number of additional drawbacks. For one, transgenic animals are usually less desirable as models of tolerance because of the presence of antigens at birth. The immune system views antigens present at birth as "self" antigens and produces long term immunological tolerance to self-antigens by thymic deletion of T cells specifically immunoreactive with those antigens. In contrast, the later development of tolerance to non-self antigens by exposure of antigens in the liver has a different underlying mechanism. Therefore, animal models in which antigens are expressed at birth do not provide a good model of tolerance to non-self antigens as develops from exposure of antigens in the liver later in life. See, e.g., Waddington et al., *Curr. Opin. Mol. Ther.* (2007) 9:432-438, attached as Exhibit C.

11. Although, Wakita uses the Cre/loxP system for conditional expression of HCV antigens in the transgenic mice, genes encoding the HCV antigens are present at birth in every tissue of the animal and may be expressed at some basal level in the absence of Cre/loxP. For that matter, Wakita observes detectable levels of expression of the HCV core protein in the lung, spleen, thymus, kidney, stomach, intestines, and muscle even though Cre-mediated transgene recombination occurs only in the liver (see page 9004, col. 1). Hence, in these transgenic animals, the immune system may be exposed to HCV antigens due to leaky expression of viral genes, albeit at a low level, from birth. In contrast, the instant application provides a non-germline animal model of tolerance that more accurately mimics the natural development of tolerance during chronic HCV infection.

12. In addition, it is much more expensive and time consuming to produce numerous transgenic animals, as described by Wakita, for screening for agents that modulate

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immunological tolerance. The use of portal vein injection of nucleic acids encoding HCV immunogens, as described in the present application, greatly facilitates screening. One of skill in the art can quickly design, for example, a dozen vectors encoding different HCV epitopes and inject such vectors into the portal vein of an animal to test for the development of immunological tolerance and screen for modulators that reverse tolerance. For example, various immunodominant HCV epitopes can be rapidly screened by this method for the development of immunological tolerance and agents that relieve tolerance. Thus, this method greatly increases the ease and flexibility of screening. In contrast, producing a dozen or more transgenic animals, as described by Wakita, to test the same epitopes would entail a great deal more effort, expense, and time.

13. Based on the foregoing, namely that the combination of Gorczynski, Nakai, Wakita, and Donnelly fails to teach or suggest the method of the claimed invention, including in particular, the injection of a nucleic acid directing liver-specific expression of an HCV immunogen into the portal vein of an animal, I do not agree that the claimed invention is obvious in view of the cited references.

14. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 26th October, 2007

Signature: M Houghton
Michael Houghton, Ph.D.

CURRICULUM VITAE
MICHAEL HOUGHTON

Education

1969-1972 *B.Sc. (Honors) Biological Sciences
University of East Anglia,
Norwich, England*

1973-1977 *Ph.D. Biochemistry
King's College,
University of London,
England*

Positions

1977 – 1982 *Senior Research Investigator, Human interferon genetics, Searle Research Laboratories, Buckinghamshire, England*
1982 – 1988 *Project Leader, Non-A,Non-B Hepatitis Discovery Research, Chiron Corporation, Emeryville, CA*
1988 – 2000 *Director, Hepatitis C Research, Chiron Corporation, Emeryville, CA*
2000 – 2003 *Vice-President, Hepatitis C Research, Chiron Corporation, Emeryville, CA*
2003 – 2006 *Vice-President, Hepatitis C & Virology Research, Chiron Corporation, Emeryville, CA*
2006 – 2007 *Vice-President, Hepatitis C & Virology Research, Novartis Vaccines & Diagnostics, Inc., Emeryville, CA*
2007 - *Chief Scientific Officer, Epiphany Biosciences Inc., San Francisco, CA*

Awards

The following honors were awarded for research on hepatitis C:

1. *Co-recipient of the Karl Landsteiner Award from the American Association of Blood Banks (1992)*
2. *Co-recipient of the Robert Koch Award from Germany (1993)*
3. *Honoree of the Japanese Medical Congress (1993)*
4. *Honoree of the Triennial International Hepatitis Meeting (1993)*
5. *Co-recipient of the William Beaumont Prize from the American Gastroenterology Association (1994)*
6. *Recipient of Beatrice Bitiello Award from Italian Association for Prevention of Viral Hepatitis (1994)*
7. *Awardee of the Princess Takamatsu Cancer Research Fund(1994)*

8. *Co-recipient of the International Hepatitis Foundation Award (1998)*
9. *Co-recipient of the Hans Popper Award (Falk Foundation;1999)*
10. *Co-recipient of the Clinical Lasker Award (2000)*
11. *Co-recipient of the Dale Smith Memorial Award of the American Association of Blood Banks*

Patents

Numerous patents issued in the fields of recombinant human interferons, bacterial expression vectors, Hepatitis C and D viruses.

Publications

Transcriptional and translational control in eukaryotes

1. *"The purification and properties of hen oviduct Form B DNA-dependent RNA polymerase"* M. Houghton and R.F. Cox (1974) *Nucl. Acids Res.* 1, 299-308.
2. *"The presence of ovalbumin mRNA coding sequences in multiple restriction fragments of chicken DNA"* M.T. Doel, M. Houghton, E.A. Cook and N.H. Carey (1977) *Nucl. Acids. Res.* 4, 3701-3713.
3. *"The interaction and RNA polymerase II from wheat with supercoiled and linear plasmid templates"* D.M.J. Lilley and M. Houghton (1979) *Nucl. Acids. Res.* 6, 507-523.
4. *"The nature of the interaction of nucleosomes with eukaryotic RNA polymerase II"* D.M.J. Lilley, M.F. Jacobs and M. Houghton (1979) *Nucl. Acids Res.* 7, 377-399.
5. *"The Xenopus Oocyte as a Surrogate Secretory System"* C.D. Lane, A. Colman, T. Mohun, J. Morser, J. Champion, I. Kourides, R. Craig, S. Higgins, T.C. James, S.W. Applebaum, R.I. Ohlsson, E. Pauchas, M. Houghton, J. Matthews and B.J. Miflin (1980) *Eur. J. Biochem.* 111, 225-235.
6. *"Active multi-subunit ACh receptor assembled by translation of heterologous mRNA in Xenopus oocytes."* K. Sumikawa, M. Houghton, J.S. Emtage, B.M. Richards and E.A. Barnard (1981) *Nature* 292, 862-864.
7. *"The molecular cloning and characterization of cDNA coding for the a subunit of the acetylcholine receptor."* K. Sumikawa, M. Houghton, J.C. Smith, L. Bell, B.M. Richards and E.A. Barnard (1982) *Nucl. Acids Res.* 10, 5809-5822.
8. *"Cloning and sequence determination of the gene for the human Σ immunoglobulin & chain expressed in a myeloma cell line."* J.H. Kenten, H.V. Molgaard, M. Houghton, R.B. Derbyshire, J. Viney, L.O. Bell and H.J. Gould (1982) *P.N.A.S. (USA)* 79, 6661-6665.

9. "A study of the mRNA and genes coding for the nicotinic acetylcholine receptor." K. Sumikawa, M. Houghton, R. Miledi and E.A. Barnard (1983) in "Cell Surface Receptors," Ed. P.G. Strange pp. 249-269 (Ellis Horwood Ltd., U.K.).
10. "Molecular genetics of the acetyl choline receptor and its insertion and organization in the membrane", E.A. Barnard, M. Houghton, R. Miledi, B.M. Richards, and K. Sumikawa, *Biol. Cell* (1982) 45:383.

Molecular genetics of human fibroblast interferon

11. "The amino-terminal sequence of human fibroblast interferon as deduced primers." M. Houghton, A.G. Stewart, S.M. Doel, J.S. Emtage, M.A.W. Eaton, J.C. Smith, T.P. Patel, H.M. Lewis, A.G. Porter, J.R. Birch, T. Cartwright and N.H. Carey (1980) *Nucl. Acids Res.* 8, 1913-1931.
12. "Human interferon gene sequences" M. Houghton (1980) *Nature* 285, 536.
13. "The complete amino acid sequence of human fibroblast interferon as deduced using synthetic oligodeoxyribonucleotide primers of reverse transcriptase" M. Houghton, M.A.W. Eaton, A.G. Stewart, J.C. Smith, S.M. Doel, G.H. Catlin, H.M. Lewis, T.P. Patel, J.S. Emtage, N.H. Carey and A.G. Porter (1980) *Nucl. Acids Res.* 8, 2885-2894.
14. "The absence of introns within a human fibroblast interferon gene" M. Houghton, I.J. Jackson, A.G. Porter, S.M. Doel, G.H. Catlin, C. Barber and N.H. Carey (1981) *Nucl. Acids Res.* 9, 247-266.
15. "The cloning and expression of a human fibroblast interferon gene in bacteria." M. Houghton, S.M. Doel, G.H. Catlin, A.G. Stewart, A.G. Porter, W.C.A. Tacon, M.A.W. Eaton, J.S. Emtage and N.H. Carey (1981) *Proceedings of the Battelle International genetic Engineering Conference*, M. Keenberg, Ed. Battelle Seminars and Studies Program.
16. "Biological Properties of Human Interferon Beta 1 Synthesized in Recombinant Bacteria." K.G. McCullagh, J.A. Davies, I.S. Sim, K.M. Dawson, G.J. O'Neill, S.M. Doel, G.H. Catlin and M. Houghton (1983) *J. Interf. Res.* 3, 97-111.
17. "Active hybrids formed between human beta and alpha interferons." A.G. Porter, L.D. Bell, J.R. Adai, G.H. Catlin, J.M. Clarke, J.A. Davies, K.M. Dawson, R.B. Derbyshire, S.M. Doel, L. Dunthorne, M.E. Finlay, J. Hall, M. Houghton, C. Hynes, I.J. Lindley, M.E. Nugent, G.J. O'Neill, J.C. Smith, A.G. Stewart, W.C. Tacon, J.H. Viney, N. Warburton, P.G. Boseley and K.G. McCullagh (1985) in "The Biology of the Interferon System." Eds. Schellekens & Stewart.
18. "Novel modified beta interferons: gene cloning, expression and biological activity in bacterial extracts." A.G. Porter, L.D. Bell, J.R. Adai, G.H. Catlin, J.M. Clarke, J.A. Davies, K.M. Dawson, R.B. Derbyshire, S.M. Doel, L. Dunthorne, M.E. Finlay, J. Hall, M. Houghton, C. Hynes, I.J. Lindley, M.E. Nugent, G.J. O'Neill, J.C. Smith, A.G. Stewart, W.C. Tacon, J.H. Viney, N. Warburton, P.G. Boseley and K.G. McCullagh (1986) *DNA* 5, 137-148.

Hepatitis Delta virus

19. "The structure, sequence and expression of the hepatitis delta (D) viral genome." K.S. Wang, Q.L. Choo, A.J. Weiner, J.H. Ou, R.C. Najarian, R.M. Thayer, G.T. Mullenbach, K.J. Denniston, J.L. Gerin and M. Houghton 91986) *Nature*, 323, 508-514 (1986); *Nature*, 328, 456 (1987).
20. "The Viroid-like structure of the hepatitis delta genome: synthesis of a viral antigen in recombinant bacteria", K-S. Wang, Q-L. Choo, A.J. Weiner, J-H. Ou, K.J. Denniston, J.L. Gerin and M. Houghton (1987) in "The hepatitis delta virus and its infection", M. Rizzetto, J.L. Gerin, R.H. Purcell, Eds. pp; 71-82 (Alan Liss Inc., New York).
21. "Hepatitis delta (D) cDNA clones: Undetectable hybridization to nucleic acids from infectious Non-A, Non-B hepatitis materials and hepatitis B DNA", A.J. Weiner, K.-S. Wang, Q.-L. Choo, J.L. Gerin, D.W. Bradley, and M. Houghton (1987) *J. of Medical Virology* 21: 239-247.
22. "A single antigenomic open reading frame of the hepatitis delta virus encodes the epitope(s) of both hepatitis delta antigen polypeptides p24^D and p27^D", A.J. Weiner, Q-L. Choo, K-S. Wang, S. Govindarajan, A.G. Redeker, J.L. Gerin, and M. Houghton. *J. of Virology* (1988) p. 594-599.
23. "Towards a vaccine for the prevention of hepatitis delta virus superinfection in HBV carriers", A. Ponzetto, M. Eckart, N. D'Urso, F. Negro, M. Silvestro, F. Bonino, K.S. Wang, D. Chien, Q.-L. Choo, and M. Houghton, *Prog. Clin. Biol. Res.* (1993) 382:207-210.
24. "The effects of using recombinant vaccinia viruses expressing either large or small HDAg to protect woodchuck hepadnavirus carriers from HDV superinfection", M.R. Eckart, C. Dong, M. Houghton, N. D'Urso, and A. Ponzetto, *Prog. Clin. Biol. Res.* (1993) 382:201-205.
25. "Human CD4+ T-cell response to hepatitis delta virus: identification of multiple epitopes and characterization of T-helper cytokine profiles", R. Nisini, M. Paroli, D. Accapezzato, F. Bonino, F. Rosina, T. Santantonio, F. Sallusto, A. Amoroso, M. Houghton, and V. Barnaba, *J. Virol.* (1997) 71:2241-2251.

Non-A, non-B hepatitis/Hepatitis C virus

26. "Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome", Q-L. Choo, G. Kuo, A.J. Weiner, L.R. Overby, D.W. Bradley, and M. Houghton, *Science* (1989) 244:, pp. 359-362.
27. "An Assay for Circulating Antibodies to a Major Etiologic Virus of Human Non-A, Non-B Hepatitis." G. Kuo, Q.-L. Choo, H.J. Alter, G.I. Gitnick, A.G. Redeker, R.H. Purcell, T. Miyamura, J.L. Dienstag, M.J. Alter, C.E. Stevens, G.E. Tegtmeier, F. Bonino, M. Colombo, W.-S. Lee, C. Kuo, K. Berger, J.R. Shuster, L.R. Overby, D.W. Bradley, M. Houghton *Science* (1989) 244:362-364.
28. "Hepatitis C virus antibodies among risk groups in Spain", J.I. Esteban, L. Viladomiu, A. Bonzalez, M. Roget, J. Genesca, J. Guardia, R. Esteban, J.C. Lopez-Talavera, J.M. Hernandez, V. Vargas, M. Buti, G. Kuo, Q.-L. Choo and Michael Houghton, *Lancet* (1989) p. 294-296.

29. "Anti-Hepatitis C antibodies and non-A, non-B post-transfusion hepatitis in the Netherlands", C.L. Van Der Poel, H.W. Ressink, P.N. Lelie, A. Leentvaar-Kuypers, Q.-L. Choo, G. Kuo, M. Houghton, (1989) *The Lancet*, 2:297-298.
30. "Hepatitis C virus HCV: A causative agent of cryptogenic cirrhosis CC among Cubans", T. Parker, M. de Medina, L. Jeffers, R. Reddy, D. Bradley, E. Schiff, M. Houghton, Q.-L. Choo and G. Kuo, *Hepatology* (1989) 10:685.
31. "Anti-hepatitis C virus in non-A non-B patients responding and non-responding to alpha 2A interferon", G. Saracco, M. Houghton, G. Kuo, Q.-L. Choo, F. Rosina, V. Lattore, M.R. Torrani Cerenzia, L. Chiandussi, F. Bonino and M. Rizzetto, *J Hepatol* (1989) 9:219.
32. "Non-A, non-B hepatitis and antibody to hepatitis C virus." J.W. Mosley, R.D. Aach, F.B. Hollinger, C.E. Stevens, L.H. Barbosa, G.J. Nemo, P.V. Holland, W.H. Bancroft, H.J. Zimmerman, G. Kuo, Q.-L. Choo, M. Houghton *JAMA* 263, 1: 77-78
33. "Prevalence of antibodies to hepatitis C virus in Italian patients with hepatocellular carcinoma", M. Colombo, Q.-L. Choo, E. Del Ninno, N. Dioguardi, G. Kjo, M.F., Donato, M.A., Tommasini and M. Houghton, *The Lancet*, (1989) p. 1006-1008.
34. "Detection of antibody to hepatitis C virus in prospectively followed transfusion recipients with acute and chronic non-A, non-B hepatitis", H.J. Alter, R.H. Purcell, J.W. Shih, J.C. Melpolder, M. Houghton, Q.-L. Choo and G. Kuo, *New England Journal of Medicine* (1989) 321: 1494-1500.
35. "A cDNA fragment of hepatitis C virus isolated from an implicated donor of post-transfusion non-A, non-B hepatitis in Japan. K. Takeuchi, S. Boonmar, T. Katayama, Q.L. Choo, G. Kuo, A.J. Weiner, D.W. Bradley, M. Houghton, I. Saito, T. Miyamura. Nucl. Acids Res. (1989) 17, 24:10367-10372.
36. "Hepatitis C virus complementary DNA clones isolated from a single healthy carrier who was shown to be an implicated donor of post-transfusion non-A, non-B hepatitis", T. Miyamura, I. Saito, Y. Kubo, K. Takeuchi, S. Boonmar, T. Katayama, G. Kuo, Q.-L. Choo and M. Houghton, In: *Proceedings of the International Meeting on Non-A, Non-B Hepatitis, Tokyo, 1989*, Shikata, T., Purcell, R.H., Uchida, T. eds., Elsevier Science Publishers, Amsterdam.
37. "Autoimmune hepatitis is not associated with antibodies to hepatitis C virus (HCV)", J.G. McHutchison, G. Kuo, M. Houghton, Q.-L. Choo, A.G. Redeker, *Hepatology* (1989) 10:701.
38. "Intrafamilial transmission of hepatitis C virus", H. Kamitsukasa, H. Harada, M. Yakura, A. Fukuda, A. Ohbayashi, I. Saito, T. Miyamura, Q.-L. Choo, M. Houghton and G. Kuo, *Lancet* (1989) 2:987.
39. "High prevalence of antibody to hepatitis C virus in patients with primary liver carcinoma", M. Colombo, G. Kuo, Q.-L. Choo, M. Houghton, M.F. Donato, M.A. Tommasini, S. Bargiggia, A. Piva, E. Del Ninno and N. Dioguardi, *Hepatology* (1989) 10:700.
40. "High prevalence of antibody to hepatitis C virus in patients with hepatocellular carcinoma HCC", M. Colombo, G. Kuo, Q.-L. Choo, M. Houghton, M.A. Tommasini, M.G. Rumi, M.L. Dioguardi, M.F. Donato, and E. Del Ninno, *J. Hepatol.* (1989) 9:20.
41. "The role of hepatitis C virus HCV in chronic liver disease", W.N. Katkov, H. Cody, A.A. Evans, G. Kuo, Q.-L. Choo, M. Houghton and J.L. Dienstag, *Hepatology* (1989) 10:644.

42. "Elevated serum alanine aminotransferase ALT in blood donors: The contribution of hepatitis C virus HCV", W.N. Katkov, L.S. Friedman, H. Cody, A.A. Evans, G. Kuo, Q.-L. Choo, M. Houghton, C.E. Huggins, and J.L. Dienstag, *Hepatology* (1989) 10:581.
43. "Circulating antibodies to hepatitis C virus HCV: A study of 160 cases of acute and chronic NANB hepatitis", J.G. McHutchison, G. Kuo, M. Houghton, Q.-L. Choo and A.G. Redecker, *Hepatology* (1989) 10:645.
44. "Hepatitis C HCV associated idiopathic chronic hepatitis and cryptogenic cirrhosis", L. Jeffers, M. de Medina, F. Hasan, R. Reddy, T. Parker, M. Silva, L. Mendez, E. Schiff, M. Houghton, Q.-L. Choo and G. Kuo, *Hepatology* (1989) 10:644.
45. "Hepatitis C HCV associated hepatocellular carcinoma", F. Hasan, L. Jeffers, M. de Medina, R. Reddy, T. Parker, E. Schiff, M. Houghton, Q.-L. Choo, and G. Kuo, *Hepatology* (1989) 10:608.
46. "Seroepidemiology of hepatitis C virus HCV in selected population", A.A. Evans, H. Cody, G. Kuo, Q.-L. Choo, M. Houghton, W.N. Katkov and J.L. Dienstag, *Hepatology* (1989) 10:644.
47. "Blood-borne non-A, non-B hepatitis PT-NANB immunohistochemical identification of disease and hepatitis C virus-associated antigens", K. Krawczynski, G. Kuo, A. Dabisceglie, M. Houghton and D.W. Bradley, *Hepatology* (1989) 10:580.
48. "Detection of antibody to hepatitis C virus in patients with various chronic liver diseases", A.M. De Bisceglie, H. Alter, G. Kuo, M. Houghton and J.H. Hoofnagle, *Hepatology* (1989) 10:581.
49. "Distinction between chronic and self-limited forms of hepatitis C virus infection", A.M. Prince, B. Brotman, T. Huima, P. Krauledat, M. Houghton, G. Kuo, Q.-L. Kuo, In: *International Meeting on Non-A, Non-B Hepatitis, Tokyo, Japan, 1989*, Shikata, T., Purcell, R.H., Uchida, T. eds. Elsevier Science Publishers, Amsterdam, pp. 7-16.
50. "Discovery of hepatitis C virus and assay of non-A, non-B hepatitis virus", M. Houghton, *Jikken Igaku* (1990) 8:203-206.
51. "Detection of hepatitis C viral sequences in non-A, non-B hepatitis", A.J. Weiner, G. Kuo, D.W. Bradley, F. Bonino, G. Saracco, C. Lee, J. Rosenblatt, Q.-L. Choo and M. Houghton, *The Lancet* (1990) 335: 1-3.
52. "Hepatitis C virus: The major causative agent of viral non-A, non-B hepatitis", Q.-L. Choo, A.J. Weiner, L.R. Overby, G. Kuo and M. Houghton, *British Medical Bulletin* (1990) 46:423-441.
53. "Detection of antibody against antigen expressed by molecularly cloned hepatitis C virus cDNA: Application to diagnosis and blood screening for posttransfusion hepatitis", T. Miyamura, I. Saito, T. Katayama, S. Kikuchi, A. Tateda, M. Houghton, Q.-L. Choo and G. Kuo, *Proc. Natl. Acad. Sci. USA* (1990) 87:983-987.
54. "Hepatitis C virus antibodies in southern African blacks with hepatocellular carcinoma", M.C. Kew, M. Houghton, Q.-L. Choo and G. Kuo, *The Lancet* (1990) 335: 873-874.
55. "Hepatitis C virus infection in an area hyperendemic for hepatitis B and chronic liver disease: The Taiwan experience", D.-S. Chien, G.C. Kuo, J.-L. Sung, M.-Y. Lai, J.-C. Sheu, P.-J. Chen, P.-M. Yang, H.-M. Hsu, M.-H. Chang, C.-J. Chen, L.-C. Hahn, Q.-L. Choo, T.-H. Wang and M. Houghton, *J. Infect. Dis.* (1990) 162:817-822.

56. "Hepatitis C antibody and chronic liver disease in haemophilia", M. Makris, F.E. Preston, D.R. Triger, J.C.E. Underwood, Q.-L. Choo, G. Kuo and M. Houghton, *Lancet* (1990) 335:1117-1119.
57. "Epidemiology of hepatitis C virus. A preliminary study in volunteer blood donors", C.E. Stevens, P.E. Taylor, J. Pinsky, Q.-L. Choo, D.W. Bradley, G. Kuo and M. Houghton, *JAMA* (1990) 263:49-53.
58. "Hepatitis C HCV infection in hemodialysis units", L. Jeffers, G. Perez, M. de Medina, E. Schiff, C. Ortiz-Interian, J. Bourgoignie, C.A. Vaamonde, M. Houghton, Q.-L. Choo and G. Kuo, *Kidney Int.* (1990) 37:303.
59. "HCV: Immunologic and hybridization based diagnostics", A.J. Weiner, M.A. Truett, J. Rosenblatt, J. Han, S. Quan, A.J. Polito, G. Kuo, Q.-L. Choo and M. Houghton, In: *Viral Hepatitis and Liver Disease*, Hollinger, F.B., Lemon, S.M., Margolis, H.S., eds., Williams & Wilkins, 1990, Baltimore, MD, 360-363.
60. "The role of hepatitis C virus in non-B chronic liver disease", W.N. Katkov, J.L. Dienstag, H. Cody, A.A. Evans, Q.-L. Choo, M. Houghton and G. Kuo, *Archives Int. Med.*, (1991) 151:1548-1552.
61. "Antibody to hepatitis C virus in the serum of patients with chronic hepatitis", F. Oliveri, M. Baldi, M.R. Brunetto, G. Saracco, F. Rosina, M.T. Cerenzia, M. Rizzetto, M.L. Soranzo, L. Colla, P. Vallauri, G. Verme, G. Kuo, M. Houghton and F. Bonino, *European J. Gastroenterology & Hepatology* (1990) 2:347-350.
62. "Hepatitis C virus (HCV): A relative of the pestiviruses and flaviviruses", M. Houghton, K. Richman, J. Han, K. Berger, C. Lee, C. Dong, L. Overby, A. Weiner, D. Bradley, G. Kuo and Q.-L. Choo, In: *Viral Hepatitis and Liver Disease*, Hollinger, F.B., Lemon, S.M., Margolis, H.S., eds., Williams & Wilkins, 1990, Baltimore, MD, 328-333.
63. "Detection and mapping of immunologic epitopes expressed by bacterial cDNA clones of the hepatitis C virus", Q.-L. Choo, K. Berger, G. Kuo and M. Houghton, In: *Viral Hepatitis and Liver Disease*, Hollinger, F.B., Lemon, S.M., Margolis, H.S., eds., Williams & Wilkins, 1990, Baltimore, MD., 345-346.
64. "Serodiagnosis of hepatitis C viral infection using recombinant-based assays for circulating antibodies to different viral proteins", G. Kuo, Q.-L. Choo, J. Shuster, C. Kuo, K. Berger, W. S. Lee, A. Medina-Selby and M. Houghton, In: *Viral Hepatitis and Liver Disease*, Hollinger, F.B., Lemon, S.M., Margolis, H.S., eds., Williams & Wilkins, 1990, Baltimore, MD. 347-349.
65. "HCV: Immunologic and hybridization-based diagnostics", A. J. Weiner, M. A. Truett, J. Rosenblatt, J. Han, S. Quan, A. J. Polito, G. Kuo, Q.-L. Choo and M. Houghton, In: *Viral Hepatitis and Liver Disease*, Hollinger, F.B., Lemon, S.M., Margolis, H.S., eds., Williams & Wilkins, 1990, Baltimore, MD, 360-363.
66. "Molecular cloning of hepatitis C virus cDNA from plasma of an implicated donor or post-transfusion non-A, non-B hepatitis", S. Boonmar, K. Takeuchi, Y. Kubo, T. Katayama, H. Harada, A. Ohbayashi, Q.-L. Choo, G. Kuo, M. Houghton, I. Saito and T. Miyamura, In: *Viral Hepatitis and Liver Disease*, Hollinger, F.B., Lemon, S.M., Margolis, H.S., eds., Williams & Wilkins, 1990, Baltimore, MD, 371-374.

67. "Early events in hepatitis C virus infection of chimpanzees", Y. K. Shimizu, A. J. Weiner, J. Rosenblatt, D. C. Wong, M. Shapiro, T. Popkin, M. Houghton, H. J. Alter and R. H. Purcell, *Proc. Natl. Acad. Sci. USA* (1990) 87:6441-6444.
68. "Direct detection of hepatitis C viral sequences in non-A, non-B hepatitis", A.J. Weiner, G. Kuo, D.W. Bradley, F. Bonino, G. Saracco, C. Lee, J. Rosenblatt, and Q.-L. Choo, *Lancet* (1990) 335:1-3.
69. "Impact of blood-donor screening for anti-HCV versus ALT, and cofactors for infectivity of anti-HCV-positive blood", C. L. Van der Poel, H. W. Reesink, P. N. Lelie, M. Th. Cuijpers, A. Leentvaar-Kuypers, E. Bakker, P. J. Exel-Oehlers, A. Polito, M. Houghton and W. Schaasberg, In: *Viral Hepatitis and Liver Disease*, Hollinger, F.B., Lemon, S.M., Margolis, H.S., eds., Williams & Wilkins, 1990, Baltimore, MD, 427-430.
70. "Open prospective efficacy trial of anti-HCV screening of blood donors to prevent post-transfusion hepatitis: Interim report of the barcelona PTH study", J. I. Esteban, A. González, J. M. Hernández, P. Madoz, E. Muniz, J. Torras, J. Enriquez, J. Buenestado, C. Martin-Vega, C. Sánchez, R. Esteban, J. Guardia, M. Houghton and H. J. Alter, In: *Viral Hepatitis and Liver Disease*, Hollinger, F.B., Lemon, S.M., Margolis, H.S., eds., Williams & Wilkins, 1990, Baltimore, MD, 431-433.
71. "Blood-borne non-A, non-B hepatitis: Detection and identification of hepatitis C virus and disease-associated antigen HCV Ag in hepatocytes", K. Krawczynski, G. Kuo, A. Dabisceglie, D. Bradley, M. Houghton, M. Alter and J. Ebert, In: *Viral Hepatitis and Liver Disease*, Hollinger, F.B., Lemon, S.M., Margolis, H.S., eds., Williams & Wilkins, 1990, Baltimore, MD, 434-435.
72. "Use of anti-HCV determinations for diagnosis of chronic HCV infection", A. M. Prince, B. Brotman, T. Huima, P. Krauledat, M. Houghton, G. Kuo, Q.-L. Choo, A. Polito, R. Di Nello and M. J. Nelles, In: *Viral Hepatitis and Liver Disease*, Hollinger, F.B., Lemon, S.M., Margolis, H.S., eds., Williams & Wilkins, 1990, Baltimore, MD, 450-455.
73. "Role of hepatitis C virus in hepatocellular carcinoma", T. Miyamura, I. Saito, T. Yoneyama, K. Takeuchi, A. Ohbayashi, Y. Watanabe, Q.-L. Choo, M. Houghton and G. Kuo, In: *Viral Hepatitis and Liver Disease*, Hollinger, F.B., Lemon, S.M., Margolis, H.S., eds., Williams & Wilkins, 1990, Baltimore, MD, 559-562.
74. "Hepatitis C viral cDNA clones isolated from a healthy carrier donor implicated in post-transfusion non-A, non-B hepatitis", K. Takeuchi, S. Boonmar, Y. Kubo, T. Katayama, H. Harada, A. Ohbayashi, Q.-L. Choo, G. Kuo, M. Houghton, I. Saito and T. Miyamura", *Gene* (1990) 91:287-291.
75. "Prevalence of anti-HCV antibody in blood donors in the Tokyo area", J. Watanabe, K. Minegishi, T. Mitsumori, M. Ishifuji, T. Oguchi, M. Ueda, E. Tokunaga, E. Tanaka, K. Kiyosawa, S. Furuta, T. Katayama, G. Kuo, Q.-L. Choo, M. Houghton and K. Nishioka, *Vox Sang* (1990) 5980-12.
76. "Blood screening for non-A, non-B hepatitis by hepatitis C virus antibody assay", T. Katayama, S. Kikuchi, Y. Tanaka, I. Saito, T. Miyamura, Q.-L. Choo, M. Houghton and G. Kuo, *Transfusion* (1990) 30:374-376.

77. "The putative nucleocapsid and envelope protein genes of hepatitis C virus determined by comparison of the nucleotide sequences of two isolates derived from an experimentally infected chimpanzee and healthy human carriers", K. Takeuchi, Y. Kubo, S. Boonmar, Y. Watanabe, T. Katayama, Q.-L. Choo, G. Kuo, M. Houghton, I. Saito and T. Miyamura, *J. Gen. Virol.* (1990) 71:3027-3033.
78. "Cloning and sequence analysis of hepatitis B virus variants in non-A and non-B infections", D. Kremsdorf, V. Thiers, F. Garreau, H. Duclos, C. Porchon, M. Houghton, P. Tiollais and C. Brechot, *J Hepatol* (1990) 11:36.
79. "Hepatitis C virus infection is associated with the development of hepatocellular carcinoma", I. Saito, T. Miyamura, A. Ohbayashi, H. Harada, T. Katayama, S. Kikuchi, Y. Watanabe, S. Koi, M. Onji, Y. Ohta, Q.-L. Choo, M. Houghton and G. Kuo, *Proc. Natl. Acad. Sci. USA* (1990) 87:6547-6549.
80. "Hepatitis C-associated hepatocellular carcinoma", F. Hasan, L.J. Jeffers, M. De Medina, K.R. Reddy, T. Parker, E.R. Schiff, M. Houghton, Q.-L. Choo and G. Kuo, *Hepatology* (1990) 12:589-591.
81. Nucleotide sequence of core and envelope genes of the hepatitis C virus genome derived directly from human healthy carriers", K. Takeuchi, Y. Kubo, S. Boonmar, Y. Watanabe, T. Katayama, Q.-L. Choo, G. Kuo, M. Houghton, I. Saito, and T. Miyamura, *Nuc. Acids Res.* (1990) 18:4626.
82. "HCV testing in low-risk population", A.J. Weiner, M.A. Truett, J. Rosenblatt, J. Han, S. Quan, A.J. Polito, G. Kuo, Q.-L. Choo, M. Houghton and C. Agius, et al. *Lancet* (1990) 336:695.
83. "The relation of hepatitis C antibodies to acute non-A, non-B hepatitis NANBH in previously untreated hemophilic patients", M. Makris, M.S. Dewar, F.E. Preston, Q.-L. Choo, G. Kuo and M. Houghton, *Br J Haematol* (1990) 74:44.
84. "HCV infection in autoimmune chronic active hepatitis", S. Magrin, A. Craxi, P. Almasio, C. Fabiano, G. Fiorentino, U. Palazzo, G.B. Pinzello, G. Provenzano, L. Pagliaro, M. Houghton and J.H. Han, *J. Hepatol.* (1990) 11:99.
85. "Vertical transmission of hepatitis C virus detected by the polymerase chain reaction", M.M. Thaler, D.W. Landers, D.W. Wara, M. Houghton, G. Veereman-Wauters, R.L. Sweet, M. Brauer and J.H. Han, *Hepatology* (1990) 12:849.
86. "Patients with acute and chronic NANB hepatitis", J.G. McHutchison, G. Kuo, M. Houghton, Q.-L. Choo and A.G. Redeker, *Hepatology* (1990) 12:966.
87. "Antibodies to hepatitis C virus anti-HCV in alcoholic liver disease: An analysis of risk factors", R.J. Leal, J.G. McHutchison, M. Houghton, Q.-L. Choo, G. Kuo, A.G. Redeker, *Hepatology* (1990) 12:881.
88. "The role of hepatitis C virus in chronic liver disease in hemophilia", M. Makris, F.E. Preston, D.R. Triger, J.C.E. Underwood, G. Kuo, Q.-L. Choo and M. Houghton, *Br J Haematol* (1990) 74:6.
89. "Antibody to the hepatitis C virus in acute hepatitis and chronic liver diseases in Japan", K. Nishioka, J. Watanabe, S. Furuta, E. Tanaka, H. Suzuki, S. Iino, T. Tsuji, M. Yano, G. Kuo, Q.-L. Choo, M. Houghton and T. Oda, *Liver* (1991) 11:65-70.

90. "A high prevalence of antibody to the hepatitis C virus in patients with hepatocellular carcinoma in Japan", K. Nishioka, J. Watanabe, S. Furuta, E. Tanaka, S. Iino, H. Suzuki, T. Tsuji, M. Yano, G. Kuo, Q.-L. Choo, M. Houghton and T. Oda, *Cancer* (1991) 67:429-433.
91. "Hepatitis C virus is a distant relative of the flaviviruses and pestiviruses", Q.-L. Choo, J. Han, A.J. Weiner, L.R. Overby, D.W. Bradley, G. Kuo and M. Houghton, In: *Proceedings of the International Meeting on Non-A, Non-B Hepatitis, Tokyo, Japan, 1991*, Shikata, T., Purcell, R.H., and Uchida, T. eds., Elsevier Science Publishers, Amsterdam, pp. 47-52.
92. "The role of chronic viral hepatitis in hepatocellular carcinoma in the United States", A.M. Di Bisceglie, S.E. Order, J.L. Klein, J.G. Wagoner, M.H. Sjogren, G. Kuo, M. Houghton, Q.-L. Choo and J.H. Hoofnagle, *Amer. J. Gastroenterology* (1991) 86:335-338.
93. "Genetic organization and diversity of the hepatitis C virus", Q.-L. Choo, K.H. Richman, J.H. Han, K. Berger, C. Lee, C. Dong, C. Gallegos, D. Coit, A. Medina-Selby, P.J. Barr, A.J. Weiner, D.W. Bradley, G. Kuo and M. Houghton, *Proc. Natl. Acad. Sci. USA* (1991) 88:2451-2455.
94. "Variable and hypervariable domains are found in the regions of HCV corresponding to the flavivirus envelope and NS1 proteins and the pestivirus envelope glycoproteins", A.J. Weiner, M.J. Brauer, J. Rosenblatt, K.H. Richman, J. Tung, K. Crawford, F. Bonino, G. Saracco, Q.-L. Choo, M. Houghton and J.H. Han, *Virology* (1991) 180:842-848.
95. "Confirmation of hepatitis C virus infection by new four-antigen recombinant immunoblot assay", C.L. van der Poel, H.T.M. Cuypers, H.W. Reesink, A.J. Weiner, S. Quan, R. DiNello, J.J.P. van Boven, I. Winkel, D. Mulder-Folkerts, P.J. Exel-Oehlers, W. Schaasberg, A. Leentvaar-Kuypers, A. Polito, M. Houghton and P.N. Lelie, *Lancet* (1991) 337:317-319.
96. "Comparison of first and second generation anti-HCV recombinant immunoblot assay with 5' UTR PCR", H.W. Reesink, C.L. Van der Poel, A.D.D. Plaisier, J.W. Verstraten, M. Cuypers, *Transfusion* (1991) 31:57.
97. "Characterization of the terminal regions of hepatitis C viral RNA: Identification of conserved sequences in the 5' untranslated region and poly(A) tails at the 3' end", J.H. Han, V. Shyamala, K.H. Richman, M.J. Brauer, B. Irvine, M.S. Urdea, P. Tekamp-Olson, G. Kuo, Q.-L. Choo and M. Houghton, *Proc. Natl. Acad. Sci. USA* (1991) 88:1711-1715.
98. "Hepatitis C virus replication in 'autoimmune' chronic hepatitis", S. Magrin, A. Craxi, C. Fabiano, G. Fiorentino, P. Almasio, U. Palazzo, G. Pinzello, G. Provenzano, L. Pagliaro, Q.-L. Choo, G. Kuo, A. Polito, J. Han and M. Houghton, *J. Hepatology* (1991) 13:364-367.
99. "Use of a signature nucleotide sequence of Hepatitis C virus for detection of viral RNA in human serum and plasma", T.-A. Cha, J. Kolberg, B. Irvine, M. Stempien, E. Beall, M. Yano, Q.-L. Choo, M. Houghton, G. Kuo, J. H. Han and M. S. Urdea, *J. Clin. Microbiology* (1991) 29:2528-2534.
100. "Molecular virology of HCV", M. Houghton, In *Report on the Proceedings, Second International Symposium on HCV, Los Angeles, Bradley, D.W. ed., 1991, Advanced Therapeutics Communications, Secaucus, New Jersey*, pp. 2-3.
101. "Replication and antigenic expression of HCV antiviral antibody response and liver pathology in acute and chronic HCV infection", K. Krawczynski, M. Beach, L. Mimms, E. Meeks, D. Vallari, S. Taskar, G. Kuo, M. Houghton and D. Bradley, *Hepatology* (1991) 14:78.

102. "Molecular biology of the hepatitis C viruses: Implications for diagnosis, development and control of viral disease", M. Houghton, A. Weiner, J. Han, G. Kuo and Q.-L. Choo, *Hepatology* (1991) 14:381-388.
103. "Hepatitis C antibody in patients with chronic liver disease and hepatocellular carcinoma", M. Colombo, M. G. Rumi, M. F. Donato, M. A. Tommasini, E. Del Ninno, G. Ronchi, G. Kuo and M. Houghton, *Dig. Dis. Sci.* (1991) 36:1130-1133.
104. "Expression and characterization of HCV structural proteins using in-vitro translation and recombinant vaccinia viruses", K. Thudium, R. Spaete, K. Berger, Q.-L. Choo, M. Houghton and R. Ralston, *J. Cell. Biochem.* (1991) 15:92.
105. "Hepatitis C virus antibodies in acute icteric and chronic non-A, non-B hepatitis", J.G. McHutchison, G. Kuo, M. Houghton, Q.-L. Choo and A.G. Redecker, *Gastroenterology* (1991) 101:1117-1119.
106. "Hepatitis C virus markers for monitoring interferon therapy in chronic hepatitis C", G. Saracco, M. Baldi, P. L. Calvo, P. Manzini, M. Abate, E. Chiaberge, M.R. Brunetto, M. Rizzetto, D. Chien, G. Kuo, M. Houghton and F. Bonino, *Hepatology* (1991) 14:75A.
107. "Analysis of genomic variability of hepatitis C virus", H.T. Cuypers, I.N. Winkel, C.L. van der Poel, H.W. Reesink, P.N. Lelie, M. Houghton, and A. Weiner, *J. Hepatol.* (1991) 13:S15-19.
108. "Rabbit-derived anti-HD antibodies for HDAg immunoblotting", F. Rosina, A. Fabiano, A. Garripoli, A. Smedile, A. Mattalia, M.R. Eckart, M. Houghton, and F. Bonino, *J. Hepatol.* (Netherlands) (1991) 13(S4):130-133.
109. "Sequence variation in hepatitis C viral isolates", A.J. Weiner, C. Christopherson, J.E. Hall, F. Bonino, G. Saracco, K. Crawford, C.D. Marion, K.A. Crawford, S. Venkatakrishna, T. Miyamura, J. McHutchinson, T. Cuypers, and M. Houghton, *Hepatology* (1991) 13:S6-14.
110. "Hepatitis C virus antigen in hepatocytes: Immunomorphologic detection and identification", K. Krawczynski, M.J. Beach, D.W. Bradley, G. Kuo, A.M. di Bisceglie, M. Houghton, G.R. Reyes, J.P. Kim, Q.-L. Choo, and M.J. Alter, *Gastroenterology* (1992) 103:622-629.
111. "Disappearance of hepatitis C virus RNA in plasma during interferon alpha-2B treatment in hemophilia patients", D. Bresters, E.P. Mauser-Bunschoten, H.T. Cuypers, P.N. Lelie, J.H. Han, P.L. Jansen, M. Houghton, and H.W. Reesink, *Scand. J. Gastroenterol.* (1992) 27:166-168.
112. "Enhanced sensitivity of a second generation ELISA for antibody to hepatitis C virus", D. Bresters, H.T. Cuypers, H.W. Reesink, W.P. Schaasberg, C.L. van der Poel, E.P. Mauser-Bunschoten, M. Houghton, Q.-L. Choo, G. Kuo, R. Lesniewski, et al., *Vox Sang* (1992) 62:213-217.
113. "Early antihepatitis C virus response with second-generation C200/C22 ELISA", C.L. van der Poel, D. Bresters, H.W. Reesink, A.A. Plaisier, W. Schaasberg, A. Leentvaar-Kuypers, Q.-L. Choo, S. Quan, A. Polito, and M. Houghton, *Vox Sang* (1992) 62:208-212.
114. "Hepatitis Viruses", L.R. Overby and M. Houghton. In "Laboratory diagnosis of viral infections", (ed.: E.H. Lennette) (1992) Marcel Dekker, New York, pp. 403-441.
115. "Characterization of the hepatitis C virus E2/NS1 gene product expressed in mammalian cells", R.R. Spaete, D'A. Alexander, M.E. Rugroden, Q.-L. Choo, K. Berger, K. Crawford, C. Kuo, S.

Leng, C. Lee, R. Ralston, K. Thudium, J.W. Tung, G. Kuo and M. Houghton, Virol. (1992) 188:819-830.

116. "Evidence for immune selection of hepatitis C virus (HCV) putative envelope glycoprotein variants: Potential role in chronic HCV infections", A.J. Weiner, H.M. Geysen, C. Christopherson, J.E. Hall, T.J. Mason, G. Saracco, F. Bonino, K. Crawford, C.D. Marion, K.A. Crawford, M. Brunetto, P.J. Barr, Tatsuo Miyamura, J. McHutchinson, and M. Houghton, *Proc. Natl. Acad. Sci. USA* (1992) 89:3468-3472.
117. "Heterogeneity of the HCV Genome: Importance for Control of the Disease", M. Houghton, In: *Hepatitis C Virus: Scientific and Clinical Status*, Deinhardt, F., Bradley D.W., Houghton, M. eds., 1992, Advanced Therapeutics Communications, Secaucus, New Jersey, pp. 8-9.
118. "Prevalence of antibodies to hepatitis C virus among patients with cryptogenic chronic hepatitis and cirrhosis", L.J. Jeffers, F. Hasan, M. De Medina, R. Reddy, T. Parker, M. Silva, L. Mendez, E.R. Schiff, M. Manns, and M. Houghton, *Hepatology* (1992) 15:187-190.
119. "The hypervariable amino terminus of the hepatitis C virus E2/NS1 protein appears to be under immune selection", A.J. Weiner, C. Christopherson, J.E. Hall, K. Crawford, C.D. Marion, K.A. Crawford, P.J. Barr, K. Richman, G. Kuo and M. Houghton, In: *Vaccines 92*, Brown; F., Chanock, R.M., Ginsberg, H.S., Lerner, R.A. eds., Cold Spring Harbor Laboratory 1992, New York, pp 303-308.
120. "Diagnosis of hepatitis C virus (HCV) infection using an immunodominant chimeric polyprotein to capture circulating antibodies: Re-evaluation of the role of HCV in liver disease", D.Y. Chien, Q.-L. Choo, A. Tabrizi, C. Kuo, J. McFarland, K. Berger, C. Lee, J.R. Shuster, Tuan Nguyen, D.L. Moyer, M. Tong, S. Furuta, M. Omata, H. Alter, E. Schiff, L. Jeffers, M. Houghton, and G. Kuo, *Proc. Natl. Acad. Sci. USA* (1992) 89:10011-10015.
121. "Identification of the major, parenteral non-A, non-B hepatitis agent (hepatitis C virus) using a recombinant cDNA approach", Q.-L. Choo, G. Kuo, A. Weiner, K.-S. Wang, L. Overby, D. Bradley, and M. Houghton, *Sem. Liver Dis.* (1992) 12:279-288.
122. "Storage conditions of blood samples and primer selection affect the yield of cDNA polymerase chain reaction products of hepatitis C virus", H.T. Cuypers, D. Bresters, I.N. Winkel, H.W. Reesink A.J. Weiner, M. Houghton, C.L. van der Poel, and P.N. Lelie, *J. Clin. Microbiol.* (1992) 30:3220-3224.
123. "PCR: Application to hepatitis C virus (HCV) research and diagnostics", A.J. Weiner, S. Venkatakrishna, J.E. Hall, M. Houghton, and J. Han, In: *Frontiers in Virology*, Becker, Y., Darai, G. eds., Springer Verlag, New York (1992), pp 86-100.
124. "Early antihepatitis-C virus response with second generation C200/C22 ELISA", C.L. Van der Poel, D. Bresters, H.W. Reesink, A.A.D. Plaisier, W. Schaasberg, A. Leentvaar-Kuypers, Q.-L. Choo, S. Quan, A. Polito, M. Houghton, G. Kuo, P.N. Lelie, and H.T.M. Cuypers, *Vox Sanguinis* (1992) 62:208-212.
125. "Group specific sequences and conserved secondary structures at the 3 ℓ end of HCV genome and its implication for viral replication", J.H. Han and M. Houghton, *Nucl. Acids Res. (England)* (1992) 20:3520.

126. "Intrahepatic cytotoxic T lymphocytes specific for hepatitis C virus in persons with chronic hepatitis", M.J. Koziel, D. Dudley, J.T. Wong, J. Dienstag, M. Houghton, R. Ralston, and B. Walker, *J. Immunol.* (1992) 149:3339-3344.
127. "5' end-dependent translation initiation of hepatitis C viral RNA and the presence of putative positive and negative translational control elements within the 5' untranslated region", B.J. Yoo, R.R. Spaete, A.P. Geballe, M. Selby, M. Houghton, and J.H. Han, *Virology* (1992) 191:889-899.
128. "T cell response to recombinant proteins of hepatitis C virus in blood and liver of patients with different clinical courses of infection", P. Botarelli, M.R. Brunetto, A.J. Weiner, M.A. Minutello, D. Unutmaz, P. Calvo, F. Bonino, M. Houghton, and S. Abrignani, *Gastroenterology* (1993) 104:580-587.
129. "Use of recombinant HCV antigen in the serodiagnosis of hepatitis C virus infection: Significant improvement in HCV antibody detection as compared with the first generation HCV C100-3 ELISA and the synthetic peptide EIA tests", D.Y. Chien, Q.-L. Choo, A. Tabrizi, C. Kuo, J. McFarland, K. Berger, C. Lee, J.R. Shuster, T. Nguyen, D.L. Moyer, M. Tong, S. Furuta, M. Omata, C.T. Fong, G. Tegtmeier, H. Alter, E. Schiff, L. Jeffers, M. Houghton, and G. Kuo, *J. Gastroenterology and Hepatology* (1993) 8:S33-39.
130. "The hepatitis C virus encodes a serine protease involved in processing of the putative nonstructural proteins from the viral polyprotein precursor", M.R. Eckart, M. Selby, F. Masiarz, C. Lee, K. Berger, K. Crawford, C. Kuo, G. Kuo, and M. Houghton, *Biochem. and Biophys. Res. Comm.* (1993) 192:399-406.
131. "Long-term follow-up of patients with chronic hepatitis C treated with different doses of interferon- β 2b", G. Saracco, F. Rosina, M.L. Abate, L. Chiandussi, V. Gallo, E. Cerutti, A. Di Napoli, A. Solinas, A. DePlano, A. Tocco, P. Cossu, D. Chien, G. Kuo, A. Polito, A.J. Weiner, M. Houghton, G. Verme, F. Bonino, and M. Rizzetto, *Hepatology* (1993) 18:1300-1305.
132. "Hepatitis C virus (HCV)-specific cytotoxic T lymphocytes recognized epitopes in the core and envelope proteins of HCV", M.J. Koziel, D. Dudley, N. Afshal, Q.-L. Choo, M. Houghton, R. Ralston, and B.D. Walker, *J. Virol.* (1993) 67:7522-7532.
133. "Long term treatment of chronic hepatitis C with interferon alfa-2b: disappearance of HCV-RNA in a pilot study of eight haemophilia patients", D. Bresters, E.P. Mauser-Bunschoten, H.T. Cuypers, J.H. Han, P.L. Jansen, R.A. Chamuleau, M. Houghton, and H.W. Reesink, *Gut (England)* (1993) 34:S124-125.
134. "Rabbit-derived anti-HD antibodies for HDAg immunoblotting", F. Rosina, A. Fabiano, E. Maran, R. Cozzolongo, A. Smedile, G. Mazzucco, A. Garripoli, C. Costa, M.R. Eckart, and M. Houghton, *Proc. Clin. Biol. Res.* (1993) 382:189-191.
135. "Expression, identification and subcellular localization of the proteins encoded by the hepatitis C viral genome", M.J. Selby, Q.-L. Choo, K. Berger, G. Kuo, E. Glazer, M. Eckart, C. Lee, D. Chien, C. Kuo, and M. Houghton, *J. Gen. Virol.* (1993) 74:1103-1113.
136. "A unique, predominant hepatitis C virus variant found in an infant born to a mother with multiple variants", A.J. Weiner, M.M. Thaler, K. Crawford, K. Ching, J. Kansopon, D.Y. Chien, J.E. Hall, F. Hu, and M. Houghton, *J. Virol.* (1993) 67:4365-4368.

137. "Compartmentalization of T lymphocytes to the site of disease: Intrahepatic CD4+ T cells specific for the protein NS4 of hepatitis C virus in patients with chronic hepatitis C", M.A. Minutello, P. Pileri, D. Unutmaz, S. Censini, G. Kuo, M. Houghton, M.R. Brunetto, F. Bonino, and S. Abrignani, *J. Exp. Med.* (1993) 178:17-25.
138. "Antibody response to core, envelope and nonstructural hepatitis C virus antigens: Comparison of Immunocompetent and immunosuppressed patients", A.S.F. Lok, D. Chien, Q.-L. Choo, T.-M. Chan, E.K.W. Chiu, I.K.P. Cheng, M. Houghton, and G. Kuo, *Hepatology* (1993) 18:497-502.
139. "Persistence of HCV despite antibodies to both putative envelope glycoprotein", (letter), D.Y. Chien, Q.-L. Choo, R. Ralston, R. Spaete, M. Tong, M. Houghton, and G. Kuo, *Lancet* (1993) 342:933.
140. "Hepatitis C virus-specific CTL responses in the liver of chimpanzees with acute and chronic hepatitis C", A.L. Erickson, M. Houghton, Q.-L. Choo, A.J. Weiner, R. Ralston, E. Muchmore, and C.M. Walker, *J. Immunol.* (1993) 151:4189-4199.
141. "Characterization of hepatitis C virus envelope glycoprotein complexes expressed by recombinant vaccinia viruses", R. Ralston, K. Thudium, K. Berger, C. Kuo, B. Gervase, J. Hall, M. Selby, G. Kuo, M. Houghton, and Q.-L. Choo, *J. Virol.* (1993) 67:6753-6761.
142. "T-lymphocyte response to hepatitis C virus in different clinical courses of infection", P. Botarelli, M.R. Brunetto, M.A. Minutello, P. Calvo, D. Unutmaz, A.J. Weiner, Q.-L. Choo, J.R. Shuster, G. Kuo, F. Bonino, M. Houghton, and S. Abrignani, *Gastroenterology* (1993) 104:580-587.
143. "Discovery of the genome of the hepatitis C virus: A general method for identifying etiologic agents of infectious disease", M. Houghton, G. Kuo, Q.-L. Choo, and D. Bradley, (1993).
144. "Vaccination of chimpanzees against infection by the hepatitis C virus", Q.-L. Choo, G. Kuo, R. Ralston, A. Weiner, D. Chien, G. Van Nest, J. Han, K. Berger, K. Thudium, C. Kuo, J. Kansopon, J. McFarland, A. Tabrizi, K. Ching, B. Moss, L.B. Cummins, M. Houghton, and E. Muchmore, *Proc. Natl. Acad. Sci. USA* (1994) 91:1294-1298.
145. "A proposed system for the nomenclature of hepatitis C viral genotypes", P. Simmonds, A. Alfredo, H.J. Alter, F. Bonino, D.W. Bradley, C. Brechot, J.T. Brouwer, S.-W. Chan, K. Chayama, D.-S. Chen, Q.-L. Choo, M. Colombo, H.T.M. Cuypers, T. Date, G.M. Dusheiko, J.I. Esteban, O.Fay, S.J. Hadziyannis, J. Han, A. Hatzakis, E.C. Holmes, H. Hotta, M. Houghton, B. Irvine, M. Kohara, J.A. Kolberg, G. Kuo, J.Y.N. Lau, P.N. Lelie, G. Maertens, F. McOmisch, T. Miyamura, M. Mizokami, A. Nomoto, A.M. Prince, H.W. Reesink, C. Rice, M. Roggendorf, S.W. Schalm, K. Shimotohno, L. Stuyver, C. Trépo, A. Weiner, P.L. Yap, and M.S Urdea, *Hepatology* (1994) 19:1321-1324.
146. "Hepatitis C Virus: Structure, protein products and processing of the polyprotein precursor", M. Houghton, M. Selby, A. Weiner, and Q.-L. Choo, In Reesink, H.W. (ed.) *Hepatitis C Virus. Curr. Stud. Hematol. Blood Trans. Karger, Basel, Switzerland* (1994) 61:1-11.
147. "The Hepatitis C Virus: Genetic organization, persistence, and vaccine strategies", M. Houghton, Q.-L. Choo, G. Kuo, R. Ralston, M. Selby, A. Weiner, D. Chien, J. Han, C. Walker, S. Abrignani, M. Koziel, B. Walker, L. Cummins, and E. Muchmore, In Nishioka, K., Suzuki,

- H., Mishiro, S., Oda, T. (eds.) *Viral Hepatitis and Liver Disease*. Springer-Verlag, Tokyo (1994) pp. 33-37.
148. "HCV-positive, HIV-1-negative mothers transmit HCV", A.J. Weiner, M.M. Thaler, K. Crawford, J. Kansopon, K. Ching, J.E. Hall, F. Hu, D. Chien, and M. Houghton, In Nishioka, K., Suzuki, H., Mishiro, S., Oda, T. (eds.) *Viral Hepatitis and Liver Disease*. Springer-Verlag, Tokyo (1994) pp. 463-467.
 149. "Peptide immunogen mimicry of putative E1 glycoprotein-specific epitopes in hepatitis C virus", R. Ray, A. Khanna, L.M. Lagging, K. Meyer, Q.-L. Choo, R. Ralston, M. Houghton, and P.R. Becherer, *J. Virol.* (1994) 68:4420-4426.
 150. "Complex processing and protein: Protein interactions in the E2:NS2 region of HCV", M.J. Selby, E. Glazer, F. Masiarz, and M. Houghton, *Virology* (1994) 204:114-122.
 151. "Hepatitis C virus markers in patients with long-term biochemical and histological remission of chronic hepatitis", G. Saracco, M.L. Abate, M. Baldi, P.L. Calvo, P. Manzini, M.R. Brunetto, F. Oliveri, G. Kuo, D. Chien, and M. Houghton, et al., *Liver* (1994) 14:65-70.
 152. "Distinct subtypes of hepatitis C virus defined by antibodies directed to the putative core, NS4, and NS5 region polypeptides", D.Y. Chien, J. McFarland, A. Tabrizi, C. Kuo, M. Houghton, and G. Kuo, *Viral Hepatitis and Liver Disease* (K. Nishioka, H. Suzuki, S. Mishiro, T. Oda eds.) Springer-Verlag, Tokyo (1994) pp. 320-324.
 153. "Transfection of a differentiated human hepatoma cell line (Huh7) with in vitro-transcribed hepatitis C virus (HCV) RNA and establishment of a long-term culture persistently infected with HCV", B.J. Yoo, M.J. Selby, J. Choe, B.S. Suh, S.H. Choi, J.S. Joh, G.J. Nuovo, H.-S. Lee, M. Houghton, and J.H. Han, *J. Virol.* (1995) 69:32-38.
 154. "Cytotoxic T lymphocyte response to hepatitis C virus—Derived peptides containing the HLA A2.1 binding motif", A. Cerny, J.G. McHutchison, C. Pasquinelli, M.E. Brown, M.A. Brothers, B. Grabscheid, P. Fowler, M. Houghton, and F.V. Chisari, *J. Clin. Invest.* (1995) 95:521-530.
 155. "Induction in vitro of a primary human antiviral cytotoxic T cell response", A. Cerny, P. Fowler, M.A. Brothers, M. Houghton, H.J. Schlicht, and F.V. Chisari, *Eur. J. Immunol.*, (1995) 25:627-630.
 156. "Persistent hepatitis C virus infection in a chimpanzee is associated with emergence of a cytotoxic T lymphocyte escape variant", A. Weiner, A.L. Erickson, J. Kansopon, K. Crawford, E. Muchmore, A.L. Hughes, M. Houghton, and C.M. Walker, *Proc. Natl. Acad. Sci. USA* (1995) 92:2755-2759.
 157. "Induction in vitro of a primary human antiviral cytotoxic T cell response", A. Cerny, P. Fowler, M.A. Brothers, M. Houghton, H.J. Schlicht, and F.V. Chisari, *Eur. J. Immunol.* (1995) 25:627-630.
 158. "Prospects for prophylactic and therapeutic hepatitis C virus vaccines", M. Houghton, Q.-L. Choo, G. Kuo, A. Weiner, D. Chien, R. Ralston, M. Urdea, B. Moss, R. Purcell, L. Cummins, and E. Muchmore, *Princess Takamatsu Symposium*, Princeton Scientific Publishing Co., Inc., (1995) 25: pp. 237-243.
 159. "Association of cytotoxic T lymphocyte (CTL) escape mutations with persistent hepatitis C virus (HCV) infection", A.J. Weiner, A.L. Erickson, J. Kansopon, K. Crawford, E. Muchmore,

- M. Houghton, and C.M. Walker. Princess Takamatsu Symposium, Princeton Scientific Publishing Co., Inc., (1995) 25: pp. 227-235.*
160. "Immune responses to plasmid DNA encoding the hepatitis C virus core protein", L.M. Lagging, K. Meyer, D. Hoft, M. Houghton, R.B. Belshe, and R. Ray, *J. Virol.* (1995) 69:5859-5863.
 161. "HLA class I-restricted cytotoxic T lymphocytes specific for hepatitis C virus: Identification of multiple epitopes and characterization of patterns of cytokine release", M.J. Koziel, D. Dudley, N. Afdhal, A. Grakoui, C. Rice, Q.-L. Choo, M. Houghton, and B.D. Walker, *J. Clin. Invest.* (1995) 96:2311-2321.
 162. "Lack of antibodies to the envelope glycoproteins of hepatitis C virus in immunoglobulin preparations from screened donors", M. Piazza, D. Chien, S. Quan, and M. Houghton, *Boll. Soc. Ital. Biol. Sper.* (1996) 72:69-70.
 163. "Hepatitis C Viruses", M. Houghton, in *Fields Virology* (B.N. Fields, D.M. Knipe, P.M. Howley, et al. eds.), Lippincott-Raven Publishers, Philadelphia, (1996) pp. 1035-1058.
 164. "A quantitative test to estimate neutralizing antibodies to the hepatitis C virus: cytofluorimetric assessment of envelope glycoprotein 2 binding to target cells", D. Rosa, S. Campagnoli, C. Moretto, E. Guenzi, L. Cousens, M. Chin, C. Dong, A.J. Weiner, J.Y. Lau, Q.-L. Choo, D. Chien, P. Pileri, M. Houghton, and S. Abrignani, *Proc. Natl. Acad. Sci. USA* (1996) 93:1759-1763.
 165. "Different clinical behaviors of acute hepatitis C virus infection are associated with different vigor of the anti-viral cell-mediated immune response", G. Missale, R. Bertoni, V. Lamonaca, A. Valli, M. Massari, C. Mori, M.G. Rumi, M. Houghton, F. Fiaccadori, and C. Ferrari, *J. Clin. Invest.* (1996) 98:706-714.
 166. "Different clinical behaviors of acute hepatitis virus infection are associated with different vigor of the anti-viral cell-mediated immune response", G. Missale, R. Bertoni, V. Lamonaca, A. Valli, M. Massari, C. Mori, M.G. Rumi, M. Houghton, F. Fiaccadori, and C. Ferrari, *J. Clin. Invest.* (1996) 98:706-714.
 167. "Quantitative analysis of the peripheral blood cytotoxic T lymphocyte response in patients with chronic hepatitis C virus infection", B. Rehermann, K.M. Chang, J.G. McHutchinson, R. Kokka, M. Houghton, and F.V. Chisari, *J. Clin. Invest.* (1996) 98:1432-1440.
 168. "Differential cytotoxic T-lymphocyte responsiveness to the hepatitis B and C viruses in chronically infected patients", B. Rehermann, K.M. Chang, J. McHutchinson, R. Kokka, M. Houghton, C.M. Rice, and F.V. Chisari, *J. Virol.* (1996) 70:7092-7102.
 169. "Development of an HCV vaccine", M. Houghton, Q.-L. Choo, D. Chien, G. Kuo, A. Weiner, S. Coates, L. Cousens, M. Winger, M. Selby, R. Ralston, K. Berger, C. Dong, K. Crawford, J. Kansopon, M. Chin, S. Wong, A. Tabrizi-Weight, R.H. Purcell, E. Muchmore, M. Morandi, D. Rosa, and S. Abrignani, in: *Viral Hepatitis and Liver Disease*, M. Rizzetto, R.H. Purcell, J.L. Gerin, and G. Verme (eds.), (1996) Edizioni Minerva Medica, Torino, Italy, pp. 656-659.
 170. "The role of hepatitis C virus-specific cytotoxic T lymphocytes in chronic hepatitis C", D.R. Nelson, C.G. Marousis, G.L. Davis, C.M. Rice, J. Wong, M. Houghton, and Y.N. Lau, *J. Immunol.* (1997) 158:1473-1481.

171. "Hepatitis C virus core and E2 protein expression in transgenic mice", C. Pasquinelli, J.M. Shoenberger, J. Chung, K.M. Chang, L.G. Guidotti, M. Selby, K. Berger, R. Lesniewski, M. Houghton, and F.V. Chisari, *Hepatology* (1997) 25:719-727.
172. "Immunodominant CD4⁺ T-cell epitope within nonstructural protein 3 in acute hepatitis C virus infection", H.M. Diepolder, J.-T. Gerlach, R. Zachoval, R.M. Hoffmann, M.-C. Jung, E.A. Wierenga, S. Scholz, T. Santantonio, M. Houghton, S. Southwood, A. Sette, and G.R. Pape, *J. Virol.* (1997) 71:6011-6019.
173. "Hepatitis C virus heteroduplex tracking assay for genotype determination reveals diverging genotype 2 isolates in Italian hemodialysis patients", P.L. Calvo, J. Kansopon, K. Sra, S. Quan, R. DiNello, R. Guaschino, G. Calabrese, F. Danielle, M.R. Brunetto, F. Bonino, A.L. Massaro, A. Polito, M. Houghton, and A.J. Weiner, *J. Clin. Microbiol.* (1998) 36:227-233.
174. "Resolution of acute hepatitis C virus (HCV) infection in chimpanzees is associated with a multi-specific, class I MHC restricted cytotoxic T lymphocyte response and not anti-HCV antibodies", S. Cooper, A.E. Erickson, D. Chien, A. Weiner, H. Houghton, P. Parham, and C.M. Walker, (1997) *in preparation*.
175. "Liver-derived CTL in hepatitis C virus infection: Breadth and specificity of responses in a cohort of persons with chronic infection", D.K.H. Wong, D.D. Dudley, N.H. Afshar, J. Dienstag, C.M. Rice, *J. Immunol.* (1998) 160:1479-1488.
176. "High titers of antibodies inhibiting the binding of envelope to human cells correlate with natural resolution of chronic hepatitis C", K. Ishii, D. Rosa, Y. Watanabe, T. Katayama, H. Harada, C. Wyatt, K. Kiyosawa, H. Aizaki, Y. Matsuura, M. Houghton, S. Abrignani, and T. Miyamura, *Hepatology* (1998) 28:1117-1120.
177. "Binding of hepatitis C virus to CD81", P. Pileri, Y. Uematsu, S. Campagnoli, G. Galli, F. Falugi, R. Petracca, A.J. Weiner, M. Houghton, D. Rosa, G. Grandi, Abrignani S. *Science* (1998) 282:938-941.
178. "Hepatitis C virus envelope glycoprotein E1 originates in the endoplasmic reticulum and requires cytoplasmic processing for presentation by class I MHC molecules", M. Selby, A. Erickson, C. Dong, S. Cooper, P. Parham, M. Houghton, and C.M. Walker. *J. Immunol.* (1999) 162:669-676.
179. Cooper S, Erickson AL, Adams EJ, Kansopon J, Weiner AJ, Chien DY, Houghton M, Parham P, Walker CM. Analysis of a successful immune response against hepatitis C virus. *Immunity* 1999; 10:439-449.
180. Colombatto, P., Brunetto, M. R., Kansopon, J., Oliveri, F., Maina, A., Aragon, U., Bortoli, M. L., Scatena, F., Baicchi, U., Houghton, M., Bonino. F. and Weiner, A. J. High prevalence of G1 and G2 TT-virus infection in subjects with high and low blood exposure risk: identification of G4 isolates in Italy. (1999) *Journal of Hepatology* 31: 990-996.
181. Abrignani S., Houghton M. & Hsu H.H. (1999) Perspectives for a vaccine against hepatitis C virus. *J Hepatol*, 31 Suppl 1, 259.
182. Hsu H.H., Abrignani S. & Houghton M. (1999) Prospects for a hepatitis C virus vaccine. *Clin Liver Dis*, 3, 901.

183. Liberman E., Fong Y.L., Selby M.J., Choo Q.L., Cousens L., Houghton M. & Yen T.S. (1999) Activation of the grp78 and grp94 promoters by hepatitis C virus E2 envelope protein. *J Virol*, 73, 3718.
184. Mustilli A.C., Izzo E., Houghton M. & Galeotti C.L. (1999) Comparison of secretion of a hepatitis C virus glycoprotein in *Saccharomyces cerevisiae* and *Kluyveromyces lactis*. *Res Microbiol*, 150, 179.
185. Rosen H.R., Hinrichs D.J., Gretch D.R., Koziel M.J., Chou S., Houghton M., Rabkin J., Corless C.L. & Bouwer H.G. (1999) Association of multispecific CD4(+) response to hepatitis C and severity of recurrence after liver transplantation. *Gastroenterology*, 117, 926.
186. Allander T., Drakenberg K., Beyene A., Rosa D., Abrignani S., Houghton M., Widell A., Grillner L. & Persson M.A. (2000) Recombinant human monoclonal antibodies against different conformational epitopes of the E2 envelope glycoprotein of hepatitis C virus that inhibit its interaction with CD81. *J Gen Virol*, 81, 2451.
187. Alter H.J. & Houghton M. (2000) Clinical Medical Research Award. Hepatitis C virus and eliminating post-transfusion hepatitis. *Nat Med*, 6, 1082.
188. Fan X.G., Tang F.Q., Yi H., Liu W.E., Houghton M. & Hu G.L. (2000) Effect of IL-12 on T-cell immune responses in patients with chronic HCV infection. *Apmis*, 108, 531.
189. Heile J.M., Fong Y.L., Rosa D., Berger K., Saletti G., Campagnoli S., Bensi G., Capo S., Coates S., Crawford K., Dong C., Wninger M., Baker G., Cousens L., Chien D., Ng P., Archangel P., Grandi G., Houghton M. & Abrignani S. (2000) Evaluation of hepatitis C virus glycoprotein E2 for vaccine design: an endoplasmic reticulum-retained recombinant protein is superior to secreted recombinant protein and DNA-based vaccine candidates. *J Virol*, 74, 6885.
190. Houghton M. (2000) Strategies and prospects for vaccination against the hepatitis C viruses. *Curr Top Microbiol Immunol*, 242, 327.
191. Lee A.Y., Manning W.C., Arian C.L., Polakos N.K., Barajas J.L., Ulmer J.B., Houghton M. & Paliard X. (2000) Priming of hepatitis C virus-specific cytotoxic T lymphocytes in mice following portal vein injection of a liver-specific plasmid DNA. *Hepatology*, 31, 1327.
192. Lee A.Y., Polakos N.K., Otten G.R., Ulmer J.B., Houghton M. & Paliard X. (2000) Quantification of the number of cytotoxic T cells specific for an immunodominant HCV-specific CTL epitope primed by DNA immunization. *Vaccine*, 18, 1962.
193. Petracca R., Falugi F., Galli G., Norais N., Rosa D., Campagnoli S., Burgio V., Di Stasio E., Giardina B., Houghton M., Abrignani S. & Grandi G. (2000) Structure-function analysis of hepatitis C virus envelope-CD81 binding. *J Virol*, 74, 4824.
194. Schirren C.A., Jung M.C., Gerlach J.T., Worzfeld T., Baretton G., Mamin M., Hubert Gruener N., Houghton M. & Pape G.R. (2000) Liver-derived hepatitis C virus (HCV)-specific CD4(+) T cells recognize multiple HCV epitopes and produce interferon gamma. *Hepatology*, 32, 597.
195. Bassett S.E., Guerra B., Brasky K., Miskovsky E., Houghton M., Klimpel G.R. & Lanford R.E. (2001) Protective immune response to hepatitis C virus in chimpanzees rechallenged following clearance of primary infection. *Hepatology*, 33, 1479.

196. Erickson A.L., Kimura Y., Igarashi S., Eichelberger J., Houghton M., Sidney J., McKinney D., Sette A., Hughes A.L. & Walker C.M. (2001) *The outcome of hepatitis C virus infection is predicted by escape mutations in epitopes targeted by cytotoxic T lymphocytes.* *Immunity*, 15, 883.
197. Merola M., Brazzoli M., Cocchiarella F., Heile J.M., Helenius A., Weiner A.J., Houghton M. & Abrignani S. (2001) *Folding of hepatitis C virus E1 glycoprotein in a cell-free system.* *J Virol*, 75, 11205.
198. Polakos N.K., Drane D., Cox J., Ng P., Selby M.J., Chien D., O'Hagan D.T., Houghton M. & Paliard X. (2001) *Characterization of hepatitis C virus core-specific immune responses primed in rhesus macaques by a nonclassical ISCOM vaccine.* *J Immunol*, 166, 3589.
199. Schirren C.A., Jung M.C., Worzfeld T., Mamin M., Baretton G., Gerlach J.T., Gruener N.H., Zachoval R., Houghton M., Rau H.G. & Pape G.R. (2001) *Hepatitis C virus-specific CD4+ T cell response after liver transplantation occurs early, is multispecific, compartmentalizes to the liver, and does not correlate with recurrent disease.* *J Infect Dis*, 183, 1187.
200. Tseng C.T., Miskovsky E., Houghton M. & Klimpel G.R. (2001) *Characterization of liver T-cell receptor gammadelta T cells obtained from individuals chronically infected with hepatitis C virus (HCV): evidence for these T cells playing a role in the liver pathology associated with HCV infections.* *Hepatology*, 33, 1312.
201. Weiner A.J., Paliard X., Selby M.J., Medina-Selby A., Coit D., Nguyen S., Kansopon J., Arian C.L., Ng P., Tucker J., Lee C.T., Polakos N.K., Han J., Wong S., Lu H.H., Rosenberg S., Brasky K.M., Chien D., Kuo G. & Houghton M. (2001) *Intrahepatic genetic inoculation of hepatitis C virus RNA confers cross-protective immunity.* *J Virol*, 75, 7142.
202. Legrand E., Neau D., Galperine T., Trimoulet P., Moreau JF., Pitard V., Lacut JY., Ragnaud JM., Dupon M., Le Bail B., Bernard N., Schvoerer E., Houghton M., Fleury H., Lafon ME. *CD4 T lymphocyte proliferative responses to hepatitis C virus (HCV) antigens in patients coinfecte*d with HCV and human immunodeficiency virus who responded to anti-HCV treatment. (2002) *J Infect Dis*, 186, 302.
203. Masciopinto F., Freer G., Burgio VL., Levy S., Galli-Stampino L., Bendinelli M., Houghton M., Abrignani S., Uematsu Y. *Expression of human CD81 in transgenic mice does not confer susceptibility to hepatitis C virus infection.* (2002) *Virology*, 304, 187.
204. Shoukry N.H., Grakoui A., Houghton M., Chien D.Y., Ghrayeb J., Reimann K.A. & Walker C.M. (2003) *Memory CD8+ T cells are required for protection from persistent hepatitis C virus infection.* *J Exp Med*, 197, 1645.
205. M.Y. Seo, S. Abrignani, M. Houghton and J.H. Han. *Small interfering RNA-mediated inhibition of hepatitis C virus replication in the human hepatoma cell line Huh-7* *J Virol*. 77 (2003) 810-812.
206. Masciopinto F., Giovani C., Campagnoli S., Galli-Stampino L., Colombatto P., Brunetto M., Yen TS, Houghton M., Pileri P., Abrignani S. *Association of hepatitis C virus envelope proteins with exosomes.* (2004) *Eur J Immunol*, 34, 2834.

207. Houghton M & Abrignani S. Vaccination against the hepatitis C viruses (2004) in New generation Vaccines 3^d Edition. Levine MM, Kaper JB, Rappuoli R, Liu M, Good MF, Eds (Marcel Dekker Inc., New York) pp.593-606
208. O'Hagan DT, Singh M, Dong C, Ugozzoli M, Berger K, Glazer E, Selby M, Wininger M, Ng P, Crawford K, Paliard X, Coates S, Houghton M. Cationic microparticles are a potent delivery system for a HCV DNA vaccine. (2004) Vaccine, 23, 672.
209. Coates S, Choo Q-L, Kuo G, Crawford K, Dong C, Wininger M, Weiner A, Abrignani S & Houghton M. Hepatitis C in Vaccines : Preventing Disease & Protecting Health (2004) De Quadros CA, Ed (Pan American Health Organisation) pp. 150 - 156
210. Brazzoli M, Helenius A, Foung SK, Houghton M, Abrignani S, Merola M. Folding and dimerization of hepatitis C virus E1 and E2 glycoproteins in stably transfected CHO cells. (2005) Virology, 332, 438.
211. Coates S, Choo Q-L, Kuo G, Crawford K, Dong C, Wininger M, Weiner A, Berger K, Wong S, Ralston R, Morandi M, Pileri P, Rosa D, Muchmore E, Mahoney J, Brasky K, Purcell R, Abrignani S & Houghton, M. Protection of chimpanzees against heterologous 1a viral challenge using a gpE1/gpE2 heterodimer vaccine (2005) in Proceedings of the 11th International Symposium on Viral Hepatitis & Liver Disease (eds Jilbert AR, Grgacic EVL, Vickery K, Burrell CJ, Cossart YE) pp 118-123 (Australian Center for Hepatitis Virology)
212. Nattermann J, Schneiders AM, Leifeld L, Langhans B, Schulz M, Inchauspe G, Matz B, Brackmann HH, Houghton M, Sauerbruch T, Spengler U. Serum antibodies against the hepatitis C virus E2 protein mediate antibody-dependent cellular cytotoxicity (ADCC). (2005) J Hepatol, 42, 499.
213. Houghton M, Abrignani S. Prospects for a vaccine against the hepatitis C virus. (2005) Nature, 436, 961.
214. Rosa D, Saletti G, De Gregorio E, Zorat F, Comar C, D'Oro U, Nuti S, Houghton M, Barnaba V, Pozzato G, Abrignani S. Activation of naive B lymphocytes via CD81, a pathogenic mechanism for hepatitis C virus-associated B lymphocyte disorders. (2005) PNAS, 102, 18544.
215. Vajdy M, Selby M, Medina-Selby A, Coit D, Hall J, Tandeske L, Chien D, Hu C, Rosa D, Singh M, Kazzaz J, Nguyen S, Coates S, Ng P, Abrignani S, Lin Y-L, Houghton M, O'Hagan D. Hepatitis C virus polyprotein vaccine formulations capable of inducing broad antibody and cellular immune responses (2006) J Gen Virol, 87, 2253

HEPATIC T CELLS AND LIVER TOLERANCE

Ian Nicholas Crispe

The T-cell biology of the liver is unlike that of any other organ. The local lymphocyte population is enriched in natural killer (NK) and NKT cells, which might have crucial roles in the recruitment of circulating T cells. A large macrophage population and the efficient trafficking of dendritic cells from sinusoidal blood to lymph promote antigen trapping and T-cell priming, but the local presentation of antigen causes T-cell inactivation, tolerance and apoptosis. These local mechanisms might result from the need to maintain immunological silence to harmless antigenic material in food. The overall bias of intrahepatic T-cell responses towards tolerance might account for the survival of liver allografts and for the persistence of some liver pathogens.

SINUSOID

A blood-filled space that lacks the anatomy of a capillary. Sinusoids generally contain slow-flowing blood, which facilitates cellular interactions. Such vessels are found in the bone marrow and in the liver.

The liver is an organ in which blood from the intestines, which is rich in bacterial products and in mainly harmless food-derived antigens, interacts with the circulating T-cell pool. The constitutive presence of non-self and microbial molecules imposes constraints on immune responses that are generated in the liver, and there might be distinctive control mechanisms that determine whether antigen encounter will result in immunity or tolerance.

Reasons to explain why the liver is often a site of immune tolerance have not been established, but one reasonable speculation is that it is because the liver is a site where harmless food antigens from the gut are processed and presented to the immune system. However, the liver is also subjected to invasion by pathogens that breach the intestinal mucosa and invade the circulation. Immune tolerance towards such invaders would not be advantageous, which indicates that liver lymphocytes must be able to switch rapidly from a tolerant to a responsive state.

Liver dendritic cells (DCs) facilitate the priming of T cells, and virus infections of the liver can generate T-cell immunity that allows the infection to be cleared, as is generally the case for hepatitis A virus in healthy humans and mouse hepatitis virus in immunocompetent mice^{1–3}. However, several infections of the liver persist despite the development of an immune response, including three that are of great epidemiological

significance: malaria, hepatitis B virus (HBV) and hepatitis C virus (HCV)^{4–6} (BOX 1). Also, early in the history of experimental transplantation, immunologists were surprised to discover that in many species, allogeneic liver grafts can be established and maintained without immunosuppression⁷. By contrast, skin, kidney and other allografts are rejected rapidly. These results raise the question of whether there is a global phenomenon of 'liver tolerance'. In support of this concept, tumours that often metastasize to the liver include malignant melanoma, which expresses well-defined tumour antigens⁸, as well as breast and lung cancers in humans. This indicates that the liver might be a site in which immunogenic tumour cells can evade immune surveillance.

To attempt to understand the control of tolerance and immunity in the liver, I discuss the anatomy of the liver blood vessels (sinusoids), which facilitate immune-cell interactions. The large macrophage population of the liver is described briefly, as are the unusual lymphocyte populations that are present in this organ. However, these considerations do not provide an easy explanation for the baseline state of T-cell tolerance, because the lymphocyte populations that are unusually abundant in the liver have a well-defined role in promoting anti-pathogen immunity. The 'professional' antigen-presenting cells (APCs) of the liver include DCs and an unusual type of vascular endothelial cell,

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doi:10.1038/nri981

POLYPROTEIN

A large protein that must be cleaved to yield many functional proteins. The ten proteins of hepatitis C virus are synthesized as a single polyprotein.

LIVER SINUSOIDAL ENDOTHELIAL CELLS

(LSECs). These cells form the lining endothelium of the hepatic sinusoids. They have unusual morphology (with many small holes and no basement membrane) and unusual properties as antigen-presenting cells (a strong predisposition towards the induction of tolerance, despite the expression of many co-stimulatory molecules).

SIEVE PLATE

A cluster of small holes (fenestrae) in a liver sinusoidal endothelial cell, which is believed to facilitate diffusion between the hepatic sinusoid and the underlying space of Disse, which is where solutes can interact with hepatocytes.

KUPFFER CELLS

The macrophages of the liver. These cells are derived from blood monocytes, and they phagocytose particles, including bacteria, that enter the liver sinusoids.

LIVER SINUSOIDAL ENDOTHELIAL CELLS (LSECs). Among these cell populations, evidence has been found for antigen-presenting function that results in T-cell inactivation, both through the induction of apoptosis and through other mechanisms.

Anatomy, shaped by function

The liver stands between the gastrointestinal tract and the systemic circulation. Blood from the gut, which is rich in food antigens, environmental toxins and bacterial products, including endotoxin (lipopolysaccharide, LPS), is collected in the hepatic portal vein (FIG. 1). This vessel is the main source of blood flowing through the liver, but approximately 20% of the incoming blood arrives in the hepatic artery. These two blood supplies mix in the hepatic sinusoids, where the blood percolates from the portal tracts to the central veins, passing between plates of hepatocytes through spaces that are lined by LSECs (FIG. 2a). This organization maximizes the exchange of molecules between the sinusoidal space and hepatocytes, allowing the liver to carry out its functions of digestion, detoxification and synthesis of plasma proteins. The sinusoidal endothelium is fenestrated, and the presence of clusters of small holes (known as SIEVE PLATES) raises the possibility that cells in the sinusoidal space might make direct contact with underlying connective tissue and hepatocytes⁹. Blood plasma, lymphocytes and DC precursors pass from the sinusoids into a sub-endothelial space, known as the space of Disse. From this space, lymph is collected, and it flows through lymphatic vessels that run in the portal tracts to the draining lymph nodes (FIG. 2b). The combination of slow blood flow, fenestrated endothelium and

lack of a discrete basement membrane distinguishes liver sinusoids from other vascular beds, and it might provide T cells that pass through the liver with unique access to tissue cells.

Kupffer cells

The liver contains a large population of resident macrophages, known as KUPFFER CELLS. These cells are derived from blood monocytes, and they are found mainly in the hepatic sinusoids. However, Kupffer cells can sometimes pass through the space of Disse and make direct contact with hepatocytes¹⁰. They are mobile and actively phagocytic, and they can be marked and observed *in vivo* through their capacity to endocytose fluorescent microspheres¹¹. Similar to other macrophages, Kupffer cells can phagocytose apoptotic cells^{12,13} and microorganisms^{14–16}. The interactions between Kupffer cells and T cells have been analysed *in vitro* using purified Kupffer cells, and *in vivo* by depletion techniques. In some studies, Kupffer cells from various species have been shown to act as effective APCs, resulting in T-cell proliferation and cytokine synthesis^{17,18}. However, Kupffer cells might be involved also in tolerance. *In vitro*, the synthesis of nitric oxide by Kupffer cells causes them to suppress T-cell activation¹⁹. *In vivo*, systemic immune tolerance occurs in response to alloantigenic leukocytes injected into the portal vein²⁰; this form of tolerance depends on Kupffer cells, because it is impaired if the Kupffer cells are depleted by treatment with gadolinium chloride²¹.

Intrahepatic lymphocytes

The liver contains an unusual population of resident lymphocytes, among which CD8⁺ T cells usually outnumber CD4⁺ T cells, and both natural killer (NK) and natural killer T (NKT) cells are enriched relative to their proportions in lymphoid tissues^{22,23}. Most of the intrahepatic CD8⁺ and CD4⁺ T cells have an activated phenotype. So, human liver CD8⁺ T cells express CD25 and CD69 (REF. 24). In mice, the liver can trap activated CD8⁺ T cells preferentially in perfusion experiments²⁵. The intrahepatic CD4⁺ T cells of mice have a CD45RB^{low} phenotype, and they synthesize both interferon-γ (IFN-γ) and interleukin-4 (IL-4)²⁶. When such activated CD4⁺ T cells are delivered by adoptive transfer, some survive in the liver for weeks. Among these cells, those with a T helper 1 (T_H1)-type effector function become non-functional, whereas T_H2-biased CD4⁺ T cells sustain their function²⁷. In addition, a subset of liver T cells express the CD45 isoform B220, which is expressed more commonly by B cells²⁸. The expression of B220 by T cells is associated with apoptosis, both *in vitro*²⁹ and in the liver³⁰.

The NK cells of rodent livers were identified first as 'pit cells', which were defined as large granular lymphocytes with cytotoxic activity against classic NK-cell targets, such as YAC1 cells³¹. These NK cells have long been known to increase in number during infection of the liver³². They express germline-encoded activating and inhibitory receptors, and they are present at an unusually high frequency among resident liver lymphocytes. During experimental liver injury induced by the injection of

Box 1 | Immune evasion by hepatitis C virus

Hepatitis C virus (HCV) is a main cause of liver disease in humans. HCV is a single-stranded RNA virus, for which a 9.5-kb messenger RNA encodes a single POLYPROTEIN. This polyprotein is cleaved by a combination of cellular and HCV-encoded peptidases into ten fragments. The World Health Organisation estimates that 170 million people are infected with HCV worldwide, and almost 4 million individuals are infected in the United States¹⁵⁰.

HCV primes the T-cell system, and CD4⁺ and CD8⁺ T-cell responses can sometimes co-exist with persistent infection^{151,152}. In both chimpanzees and humans, an early and diverse CD4⁺ and CD8⁺ T-cell response is associated with clearance of the virus^{153,154}. Sequence analysis of HCV isolates shows that HCV generates escape mutations in both of the two susceptible species^{155–157}. This is a classic type of immune evasion, which is also seen for other persistent viruses.

HCV might disable T cells by other mechanisms also. Some mutations create antagonistic peptides with the potential to inactivate T cells that are specific for the prototype HCV antigenic epitope^{157,158}. Such antagonism might explain the long-term persistence of individual virus genotypes, even in the presence of T-cell priming. In addition, HCV-specific T cells might become dysfunctional, particularly with regard to the synthesis of inflammatory cytokines. This has been termed 'stunning'¹⁵⁹. The mechanism is not known.

Two HCV structural proteins, core protein and E2, have immunosuppressive properties *in vitro*. HCV core protein suppresses T-cell activation by binding to the C1q receptor^{160,161}, and it might also promote CD95 (FAS)-induced apoptosis¹⁶². The HCV envelope protein E2 binds CD81 on natural killer (NK) cells and inhibits NK-cell function¹⁶³. The interpretation of this finding is complicated, because transgenic mice that express HCV core, E1 and E2 proteins have no evidence of global immunosuppression¹⁶⁴.

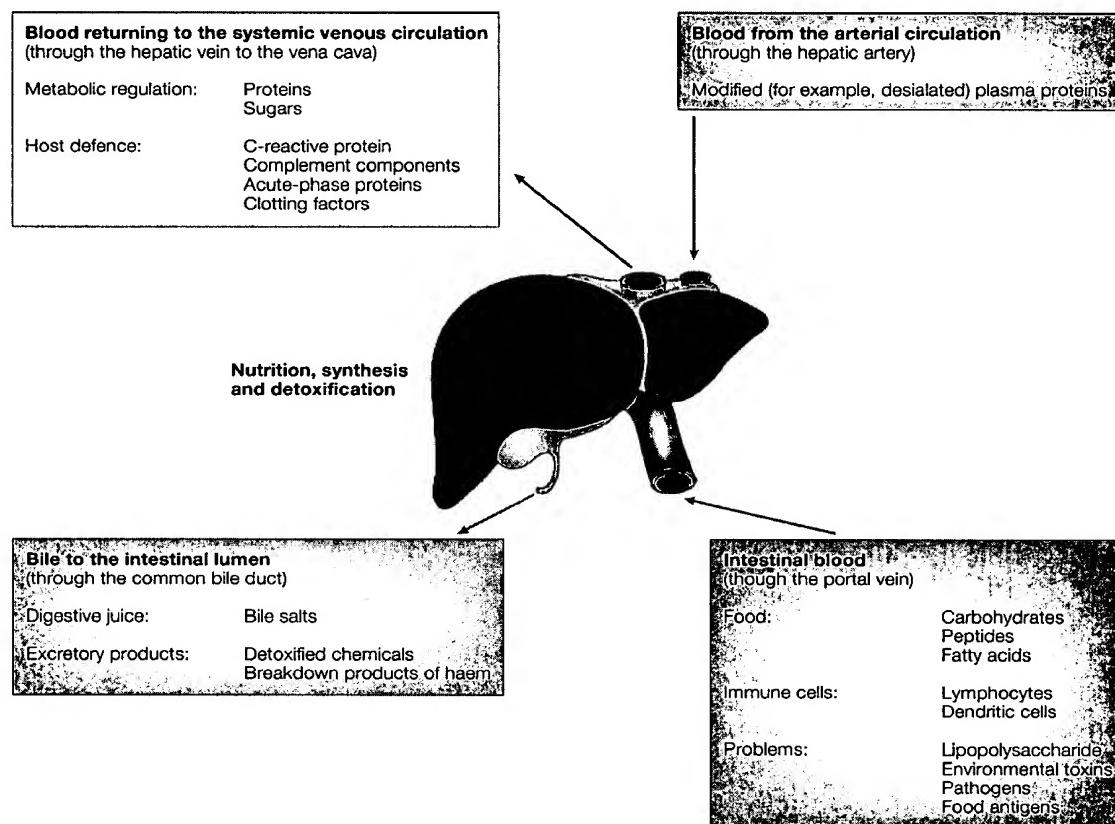


Figure 1 | The inputs, outputs and main functions of the liver. The figure shows that, in addition to its central metabolic role, and its interactions with T cells, which are summarized in this review, the liver makes an important contribution to host defence by synthesizing several defensive molecules, including complement components and clotting factors.

concanavalin A³³ and during infection of the liver with adenovirus vectors³⁴ (BOX 2), NK cells seem to have a crucial role in T-cell recruitment. During cytomegalovirus infection of mice, type I IFNs (IFN- α/β) induce synthesis of the chemokine CCL3 (also known as monocyte inflammatory protein-1 α , MIP1 α), which, in turn, is responsible for NK-cell accumulation³⁵. Lack of CCL3 compromises both NK-cell accumulation and protective immunity. The NK cells synthesize IFN- γ , which promotes secretion of the chemokine CXCL9 (monokine induced by γ -interferon, Mig), probably by hepatocytes and/or LSECs³⁶, and this is responsible for the accumulation of T cells. Therefore, liver NK cells recruit T cells through a multi-step cytokine/chemokine cascade (FIG. 3), and in so doing, they promote T-cell immunity, rather than tolerance.

The normal mouse liver is also rich in NKT cells. These cells have a complex phenotype, features of which include the expression of NK1.1 in the B6 mouse and expression of the p70 chain of the IL-2 receptor (IL-2R β)³⁷. The prototype NKT cells express, at intermediate density, a T-cell receptor (TCR) $\alpha\beta$ with specificity for the MHC class-I-like molecule CD1d. Their TCR repertoire has limited diversity; the TCR β -chain is limited to V β 8.2, V β 7 or V β 2, whereas the TCR α -chain is uniformly V α 14–Ja281, with a conserved junctional

sequence^{38,39}. These cells are present throughout the immune system, usually at a low frequency (<5% of all T cells), but they are abundant in the liver. A distinct, CD1d-independent population of NKT cells seems to be abundant at other tissue sites⁴⁰. Conversely, there are CD1d-reactive T cells that do not use the V α 14–Ja281 receptor, but that might be involved in intrahepatic pathology⁴¹. Whereas the classical NKT cells originate in the thymus⁴², an intrahepatic origin has been proposed for other NKT-like cells⁴³. The lineage relationships of these other NKT-cell populations are not known, and their identification is complicated by the fact that conventional T cells can express NK-cell markers after activation^{44,45}.

The function of NKT cells is of great interest. Early on, NKT cells were shown to secrete IL-4, and they might, therefore, be seen as 'anti-inflammatory' lymphocytes⁴⁶. In support of this idea, one well-documented function of these cells is protection from autoimmune disease in non-obese diabetic (NOD) mice, which lack these cells^{47,48}. However, *in vivo* analysis indicates that, in the liver, NKT cells have a positive role in host defence. Mice lacking the CD1d molecule have been reported to lack defences against *Mycobacterium tuberculosis* and *Borrelia burgdorferi*^{49,50}. The data on anti-protozoan immunity are conflicting, indicating an apparent role

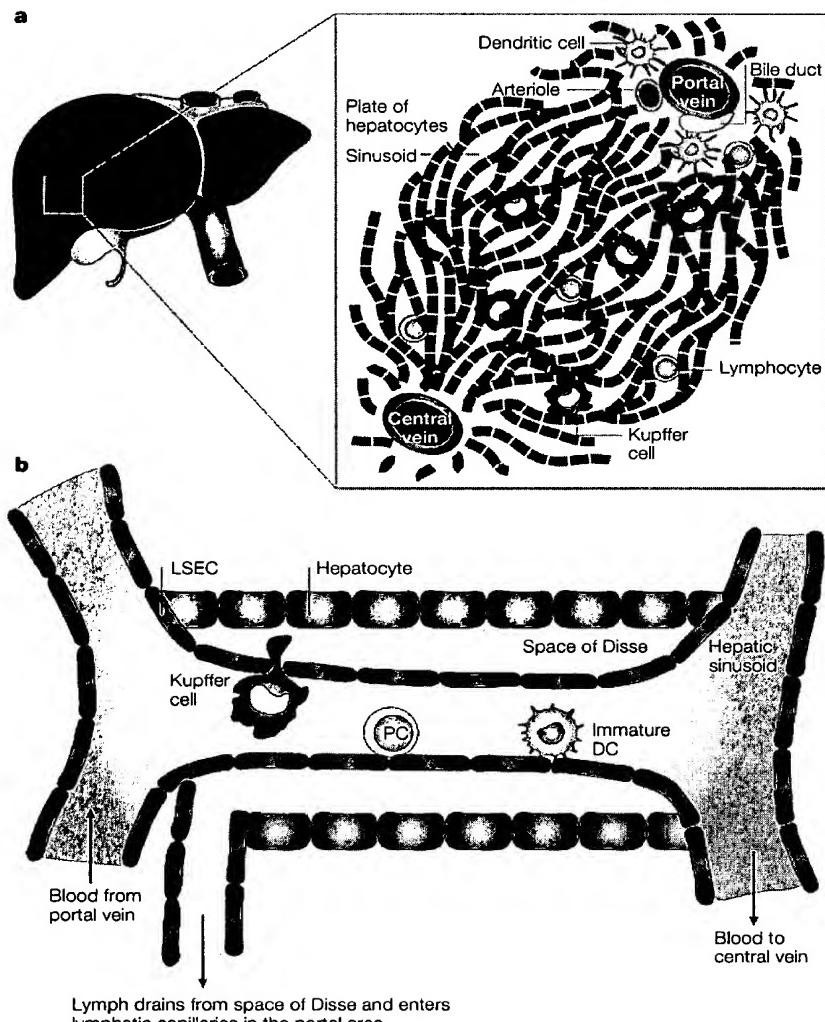


Figure 2 | The hepatic microenvironment. **a** | Diagram showing the structure of a liver lobule. The tissue is organized around vascular bundles, which are known as portal tracts. These contain a branch of the portal vein, an arteriole and a tributary of the bile duct. From the portal tracts, blood flows through a sponge-like anastomosing meshwork of sinusoids that exist between plates of hepatocytes. The sinusoids contain a large population of macrophages, known as Kupffer cells. **b** | Organization of sinusoids. The sinusoid is lined by an endothelium (liver sinusoidal endothelial cells, LSECs) that is fenestrated and lacks a basement membrane. Kupffer cells, lymphocytes (Pit cells, PCs) and immature dendritic cells (DCs) are found in the sinusoids. Kupffer cells exist mainly in the sinusoidal lumen, but they can make direct contact with hepatocytes. The sub-endothelial space, known as the space of Disse, is the region from which hepatic lymph originates.

for CD1d-reactive NKT cells in immunity to trypanosomes^{51,52}, but no role in immunity to mouse malaria⁵³. One complication in the interpretation of these experiments is that the lack of CD1d might cause effects that are not due simply to the lack of NKT cells⁵⁴. Mice that selectively lack the V α 14–J α 281 subset of NKT cells have been created, and these mice are deficient in anti-tumour immunity, which indicates that classical NKT cells have an important role in this process^{55,56}. Another tool to study the function of NKT cells is α -galactosyl ceramide (α -GalCer), a molecule derived from marine sponges that causes acute NKT-cell activation, resulting in the synthesis of IFN- γ and IL-4,

followed by deletion of the cells⁵⁷. Using α -GalCer, the activation of NKT cells *in vivo* was accompanied by the inhibition of melanoma metastases⁵⁸, induction of effective immunity to the liver stages of malaria⁵⁹ and suppression of viral-RNA synthesis in HBV-transgenic mice⁶⁰. In summary, there is strong evidence that liver NKT cells promote immunity, but no evidence to implicate these cells in liver tolerance.

The normal resident lymphocytes of the human liver have not been characterized in such detail, but similar to rodent liver lymphocytes, there is an increase in CD8 $^{+}$ T cells relative to CD4 $^{+}$ T cells, and an increase in the number of CD3 $^{+}$ cells that co-express the NK-cell marker CD56 (REF. 22). Some, but not all, of these cells express a TCR in which the V α 24 segment is joined to J α Q, forming a conserved TCR that is analogous to the V α 14–J α 281 receptor on mouse NKT cells⁶¹. As in the mouse, the caveat must apply that the expression of NK-cell markers by $\alpha\beta$ T cells could simply identify activated cells. A subset of normal human liver lymphocytes that express the markers CD2 and CD7 also express messenger RNA encoding recombination-activating gene 1 (RAG1), RAG2 and pre-T α , which indicates that these cells might be undergoing lymphocyte-receptor gene rearrangement, and which supports the concept that the liver is a site of lymphoid development⁶². However, it is also possible that these mRNAs are expressed aberrantly by haematopoietic stem cells, which are present in human liver⁶³.

During inflammation, the lymphocyte populations of the liver change. In mice, experimental infection with *Propionibacterium acnes* causes a relative increase in the number of T cells and a corresponding decrease in the number of NKT cells⁶⁴, and the same changes are seen in mice with fatty livers⁶⁵. Both of these pathologies sensitize the liver to endotoxin-mediated damage, which indicates that NKT cells might protect against such damage. In line with a tissue-sustaining function of NKT cells, the number of these cells increases during liver regeneration after partial hepatectomy⁶⁶. Infection with lymphocytic choriomeningitis virus results in two phases of T-cell infiltration, involving heavy CD8 $^{+}$ T-cell infiltration of the sinusoids, followed by CD4 $^{+}$ T-cell infiltration of the portal tracts⁶⁷. In humans, liver biopsy material provides a picture of liver lymphocytes during hepatitis. There are increases in the number of activated CD8 $^{+}$ T cells⁶⁸ and in the number of CD4 $^{+}$ T cells relative to the number of CD8 $^{+}$ T cells⁶⁹. During infection with HCV, the CD8 $^{+}$ T cells are activated (they express CD45RO) and are located mainly in the sinusoids, whereas the CD4 $^{+}$ T cells are mainly naive (they express CD45RA) and are located in the portal tracts⁷⁰. The number of T cells that express the $\gamma\delta$ TCR is also increased during viral hepatitis⁷¹. Overall, the inflamed liver shows a shift from the resting pattern of abundant NKT cells and an excess of CD8 $^{+}$ T cells over CD4 $^{+}$ T cells towards a more conventional pattern that is characteristic of other inflammatory sites. This is entirely consistent with a role for NK cells in initiating intrahepatic immune responses, but with their numbers then being diluted by the T cells that they recruit.

Interaction of the liver with systemic T cells

In the remainder of this review, I consider the interactions between the liver and conventional CD4⁺ and CD8⁺, $\alpha\beta$ T cells. Circulating T cells pass through the liver sinusoids and can interact with Kupffer cells and LSECs. Some experiments indicate further that naïve T cells might interact with hepatocytes. Antigens that are expressed in the liver might be taken up by immature DCs, and might then be presented to CD4⁺ and CD8⁺ T cells, either in lymphoid-tissue aggregates in the portal tracts or in secondary lymphoid tissues. Alternatively, antigens might be recognized *in situ* on LSECs, Kupffer cells and, possibly, hepatocytes. The outcome of antigen recognition in the liver could be full T-cell activation, immune deviation leading to the differentiation of T cells to a suppressive or regulatory phenotype, or abortive activation leading to T-cell apoptosis. In addition, the liver might sequester activated T cells in an antigen-independent manner, and the high apoptotic rate of such cells has given rise to the idea that the liver might be a 'graveyard' for systemic T cells⁷². Inflammation of the liver due to viral hepatitis is accompanied by the upregulation of expression of an extensive panel of T-cell interaction molecules, including intercellular adhesion molecule 1 (ICAM1), MHC class II molecules⁷³, vascular cell adhesion molecule 1 (VCAM1)⁷⁴, co-stimulatory molecules of the B7 family⁷⁵ and CD95 (FAS)⁷⁶. These molecules might modify cell trafficking, priming and the induction of tolerance.

Cell trafficking

In most blood vessels, leukocytes flow past the endothelium without forming adhesions. Under inflammatory conditions, selectins expressed by leukocytes interact

with addressins expressed by the endothelium to initiate 'rolling', which slows the cells and allows other adhesion molecules to become engaged⁷⁷. *In vivo* microscopy shows that the blood flow in hepatic sinusoids is slow and intermittent, facilitating interactions between blood cells and the endothelium, which might, therefore, be independent of selectin–addressin interactions⁷⁸. LSECs express several adhesion molecules, including a high density of ICAM1 and ICAM2 (REF. 79), and vascular adhesion protein 1 (VAP1)⁸⁰. The reason for this constitutive expression of adhesion molecules is unknown, but one factor might be that LPS from the intestine interacts with Toll-like receptor 4 (TLR4), which is expressed in the liver⁸¹, probably by LSECs⁸². This interaction between LPS and liver cells increases the level of expression of adhesion molecules⁸³. The ICAMs engage lymphocyte function-associated antigen 1 (LFA1) on activated T cells, whereas VAP1 binds to unknown ligand(s) on CD8⁺ T cells and on CD16⁺ cells, including NK cells. Therefore, the adhesion molecules that are expressed by LSECs give the liver a dual T-cell tropism: for activated T cells in preference to resting T cells, and for CD8⁺ T cells and NK cells in preference to CD4⁺ T cells.

In addition to lymphocytes, DCs also traffic through the liver, passing from blood in the sinusoids to the space of Disse, from there through lymphatics in the portal tracts, and ultimately to the hepatic and celiac lymph nodes⁸⁴. The migration of DCs seems to be controlled by chemokines^{85,86} (FIG. 4). During experimental infection with *P. acnes*, blood-borne DC precursors expressing CC-chemokine receptor 1 (CCR1) and CCR5 formed intrahepatic granulomas in response to CCL3. After maturation, the DCs expressed CCR7 and became responsive to CCL21 (secondary lymphoid tissue chemokine, SLC), which promoted their migration to organized lymphoid tissue⁸⁷.

Box 2 | Experimental models of immune-mediated liver damage

Injection of the lectin concanavalin A (ConA) into mice causes T-cell-dependent liver damage. This model depends on CD4⁺ T cells, interferon- γ and natural killer cells. The difficulty in interpreting this model results from the fact that ConA binds many ligands and might have unknown effects in the liver in addition to recruiting and activating T cells^{165,166}.

The infusion of non-tolerant T cells into a transgenic mouse that expresses an MHC class I molecule on hepatocytes results in transient hepatitis, accompanied by apoptosis of the T cells^{163,167}. The difficulty in interpreting this model lies in the difficulty of knowing on which cells the MHC transgene is expressed.

Transgenesis has been used also to express the entire genome of hepatitis B virus (HBV) and of hepatitis C virus in hepatocytes. Such mice develop tolerance to virus-encoded proteins, but the infusion of non-tolerant T cells causes liver inflammation^{168,169}. Interpretation of this model is complex, because although the viral antigens are targeted to hepatocytes, cross-presentation might occur on liver sinusoidal endothelial cells, Kupffer cells or dendritic cells. Virus proteins might have evolved to cause immune effects and/or to have direct cytopathic effects, which makes models of this kind both realistic and difficult to unravel. In the HBV-transgenic model, the suppression of virus RNA was due to cytokines, rather than cytotoxic mechanisms.

A simple model of 'pure' T-cell-mediated liver damage has been developed by the injection of specific antigenic peptides into T-cell-receptor-transgenic mice^{142,143}. In this model, there are no restrictions on the site of antigen presentation, which makes models that are based on the roles of different antigen-presenting cells difficult to test. Crossing the transgenic mice to a background that lacks death receptor(s) has shown their importance for this kind of liver damage.

Antigen presentation

The liver can present antigen to T cells through several mechanisms. The Kupffer cells form an antigen-trapping system, and the liver is patrolled by 'professional' APCs in the form of immature DCs. In humans, CD45⁺ cells with high-level expression of MHC class II molecules and a dendritic morphology can be seen in the portal tracts, but these cells are unusual in that they lack expression of CD11C, which is usually found on DCs⁸⁸. In mice, two populations of DC exist, which are distinguished by their expression of CD8 α . The DCs that do not express CD8 have been termed 'myeloid' DCs, whereas DCs that express CD8 α have been termed 'lymphoid' DCs. However, it is not clear that these cell types originate from distinct lineages⁸⁹. Both types of DC are present in the mouse liver, and both can differentiate into effective APCs *ex vivo*⁹⁰. Trafficking immature mouse DCs pass through the liver, and while they are in the hepatic sinusoids, these DCs are likely to interact with Kupffer cells⁹¹, in part through their expression of lectin-like carbohydrate receptors^{92,93}. This interaction might be facilitated by CCL3 synthesized by the Kupffer cells, which communicates with the DCs through their CCR1 chemokine receptors (FIG. 4).

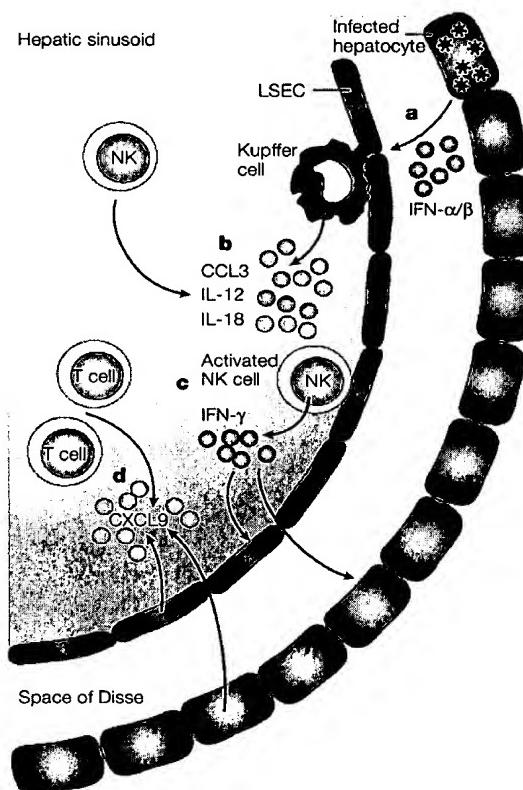


Figure 3 | The cytokine/chemokine cascade through which NK cells recruit T cells. The steps are: **a** | many cell types, including hepatocytes, synthesize type 1 interferons (IFN- α/β) in response to virus infection; **b** | Kupffer cells respond to IFN- α and IFN- β by producing CCL3, which recruits natural killer (NK) cells; **c** | NK cells, once activated by the Kupffer-cell products interleukin-12 (IL-12) and, perhaps, IL-18, produce IFN- γ , which causes tissue cells, including hepatocytes and liver sinusoidal endothelial cells (LSECs), to produce CXCL9; **d** | CXCL9 recruits T cells.

An interaction between DCs and macrophages might be important for cross-presentation — for example, of antigen derived from the phagocytosis of apoptotic Kupffer cells^{94,95} — and also for priming of the immune response to pathogens.

The *in vivo* function of DCs might lead to effective T-cell priming or to T-cell tolerance. The evidence for tolerance induction by some DCs is clear, and one important distinction between DCs that induce immunity and those that induce tolerance is that the former secrete IL-12, whereas the latter secrete IL-10. As I discuss later, secretion of IL-10 is prevalent in normal liver. Another important issue is whether the DCs that are located in the portal tracts are involved actively in antigen presentation at that site or whether they must traffic to lymph nodes first.

In addition to DCs and Kupffer cells, antigens passing through the hepatic sinusoids encounter LSECs, which form a highly distinctive endothelium. Mouse LSECs express molecules that promote antigen uptake, including the mannose receptor and the scavenger receptor, and molecules that promote antigen presentation,

including CD40, CD80 and CD86 (REF. 96). These cells are, therefore, equipped to act as APCs, and they present antigens to both CD4 $^{+}$ and CD8 $^{+}$ T cells (FIG. 5). Despite their expression of co-stimulatory molecules, the most common consequence of T-cell priming by LSECs is tolerance^{97,98}. The reason for this paradoxical behaviour is unknown. By contrast, human LSECs express CD40, but they do not seem to express CD80 or CD86 constitutively. These molecules are, however, expressed during inflammation⁹⁹. So, the APC properties of mouse and human LSECs might differ.

The sinusoidal endothelium is fenestrated. Small holes, clustered together to form sieve plates, facilitate the diffusion of metabolites backwards and forwards between the blood and hepatocytes, and scanning electron micrographs have indicated that there are also gaps between the LSECs. This creates the possibility that both naive and previously activated T cells could gain direct access to hepatocytes. This would be a unique situation, because resting naive T cells do not have access to most tissues, which are selectively populated with long-lived memory T cells^{100,101}. The extent to which such tissue access occurs in the liver is controversial. The selective accumulation of antigen-specific T cells in the livers of transgenic mice expressing either the HBV genome¹⁰² or a non-self MHC class I molecule¹⁰³ is compatible with the idea that such direct access to hepatocytes occurs in the living liver. However, these data are open to alternative interpretations. The HBV antigens could have been subject to cross-presentation, for example by LSECs, whereas the non-self MHC class I molecules could have been ectopically expressed.

T-cell tolerance due to apoptosis

The infusion of non-tolerant CD8 $^{+}$ T cells into transgenic mice that express a non-self MHC class I alloantigen on hepatocytes results in the rapid and selective accumulation of these T cells in the liver. The T cells become activated and might undergo several cell divisions, but ultimately, they die by apoptosis^{104,105} (FIG. 5b). In mice that contain antigen-specific TCR-transgenic CD8 $^{+}$ T cells and are injected systemically with soluble antigenic peptide, activation and proliferation of the T cells in lymphoid tissues is followed by the accumulation and apoptosis of these T cells in the liver^{25,30}. Finally, transplantation of an allogeneic liver into both humans and mice results in T-cell infiltration, followed by apoptosis of the infiltrating cells^{106,107}. To investigate the basis of these observations, naive T cells have been cultured with purified alloantigenic hepatocytes, which results in partial activation of the T cells, followed by their apoptosis. This is believed to be an example of a type of apoptosis known as PASSIVE CELL DEATH (PCD).

In mature T cells, PCD can occur when fully activated T cells are deprived of antigen, or of growth and survival factors such as IL-2. This seems to be a main mechanism for the clearance of expanded clones of T cells after antigen has been cleared during and after an acute infection, whereas FAS-induced apoptosis is less important in this situation¹⁰⁸. The mechanism of PCD involves loss of mitochondrial membrane integrity,

CROSS-PRESENTATION

The process by which exogenous antigens that are expressed by one cell are processed and presented by MHC class I molecules of another cell. Peptides derived from antigenic proteins are susceptible to this form of presentation, whereas MHC alloantigens are not. Dendritic cells and liver sinusoidal endothelial cells are particularly efficient at cross-presentation.

PASSIVE CELL DEATH

The death of T cells due to activation in the absence of sufficient survival signals, or when antigen is cleared and signals through the T-cell receptor cease.

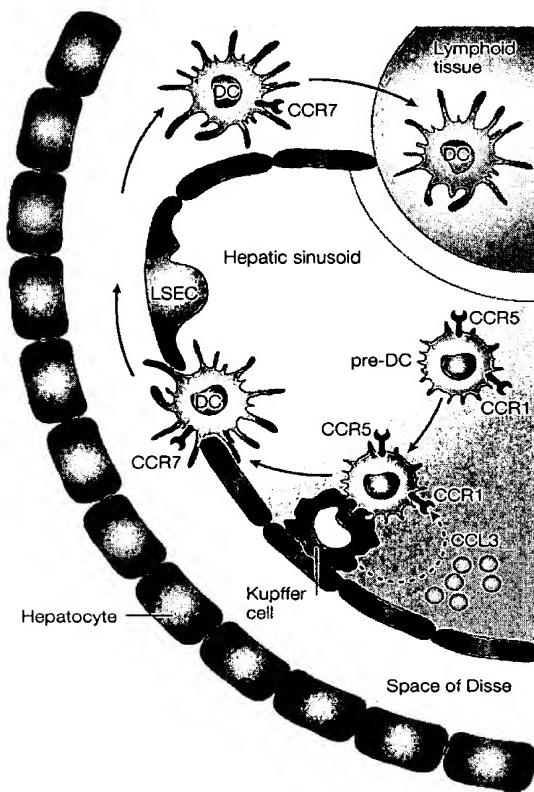


Figure 4 | Trafficking and interactions of dendritic cells in the liver, controlled by chemokines. In response to inflammatory signals, Kupffer cells secrete CCL3, which engages CC-chemokine receptor 1 (CCR1) on immature dendritic cells (pre-DCs). Maturation of these cells results in the loss of expression of CCR1 and CCR5, and the expression of CCR7. The DCs become responsive to CCL21, which is produced by lymphatic endothelia and tissue stroma, both in lymph nodes and in the portal tracts. DCs leave the hepatic sinusoids and migrate to lymphoid aggregates in the portal tracts or to lymph nodes. LSEC, liver sinusoidal endothelial cell.

the release of cytochrome *c* and the activation of pro-caspase-9 through apoptotic protease-activating factor 1 (APAF1)^{109,110}. Once activated, caspase-9 cleaves and activates pro-caspase-3 and -7, which yields the effector caspases that are responsible for the breakdown of many crucial substrates, including other caspases¹¹¹. In contrast to PCD, ACTIVATION-INDUCED CELL DEATH (AICD) occurs in fully activated T cells and is promoted by IL-2 (REFS 112,113). This process depends on the expression of death receptors, such as FAS and tumour-necrosis factor receptor 1 (TNFR1), and on activation of the death-receptor-initiated caspase cascade, which occurs in part by the downregulation of expression of the inhibitory molecule FLIP (FLICE-like inhibitory protein)¹¹⁴. After their ligation, death receptors assemble with adaptor proteins to form a death-inducing signalling complex (DISC), the effect of which is to cleave and activate pro-caspase-8 (REF. 115). Activated caspase-8 cleaves pro-caspase-3, leading to convergence of the PCD and AICD pathways¹¹¹.

ACTIVATION-INDUCED CELL DEATH (AICD). The apoptosis of fully activated T cells, mediated by ligation of death receptors — such as CD95 (FAS), tumour-necrosis factor receptor 1 (TNFR1) and TNF-related apoptosis-inducing ligand receptor (TRAILR) — on their surface.

Experiments in which hepatocytes induce the abortive activation, followed by death, of naive T cells might be examples of PCD. The addition of exogenous IL-2 inhibits the death of T cells that are co-cultured with hepatocytes, which supports the theory that this form of cell death is PCD¹⁰⁵. Mouse hepatocytes, unlike mouse LSECs, do not express CD80 and CD86 (REF. 116), which indicates that PCD might be induced by the presentation of antigen in the absence of normal co-stimulatory ligands — the same conditions that would cause anergy of a previously activated T-cell clone¹¹⁷. A role for PCD due to lack of co-stimulation in liver allograft tolerance *in vivo* was indicated by experiments in which liver-grafted mice were treated with cytotoxic T-lymphocyte antigen 4 (CTLA4)-immunoglobulin, which is an inhibitor of CD80/CD86-mediated co-stimulation. In the CTLA4-immunoglobulin-treated mice, enhanced graft survival was associated with the increased apoptosis of graft-infiltrating CD4⁺ and CD8⁺ T cells¹¹⁸.

If T cells die in the liver by PCD, death receptors such as FAS and TNFR1 would be expected to be irrelevant. One clear example contradicts this and provides evidence in favour of an AICD model. The CD8⁺ T-cell infiltrate in livers infected with a recombinant adenovirus vector was cleared by a Fas-dependent mechanism¹¹⁹. This result argues against a purely passive interpretation of T-cell death in the liver. Such AICD-based models might depend on systemic T-cell activation, because such activation induced by super-antigens results in the upregulation of expression of Fas ligand (FasL) in several tissues, including the liver¹²⁰.

How can the liver induce both PCD and AICD of CD8⁺ T cells? The answer might be that AICD depends on full T-cell activation and differentiation. In most of the models that I discuss, T-cell activation is not optimum. Examples include the transgenic expression of non-self MHC molecules on hepatocytes and a liver transplant in the absence of infection. Although the transplant procedure might result in tissue damage and some inflammation, the antigen-presenting system must not be sufficiently perturbed to change to an immunogenic state. Both of these situations, therefore, seem to result in PCD of CD8⁺ T cells. By contrast, in the adenovirus model, there was evidence for the involvement of Fas and, therefore, of AICD. So, it is probable that the liver kills CD8⁺ T cells by two mechanisms. Direct recognition of antigen on LSECs, and possibly also on hepatocytes, by naive CD8⁺ T cells results in partial activation of the T cells, followed by PCD (FIG. 5b). By contrast, recognition of liver antigens by CD8⁺ T cells in the context of an adenovirus infection results in full T-cell activation, rendering the T cells susceptible to AICD. The role of AICD in the disappearance of CD8⁺ T-cell infiltrates in other models of viral infection is unexplored, although an unusual variation of AICD might occur in liver CD8⁺ T cells that are specific for *Listeria monocytogenes*, the clearance of which depends on perforin¹²¹.

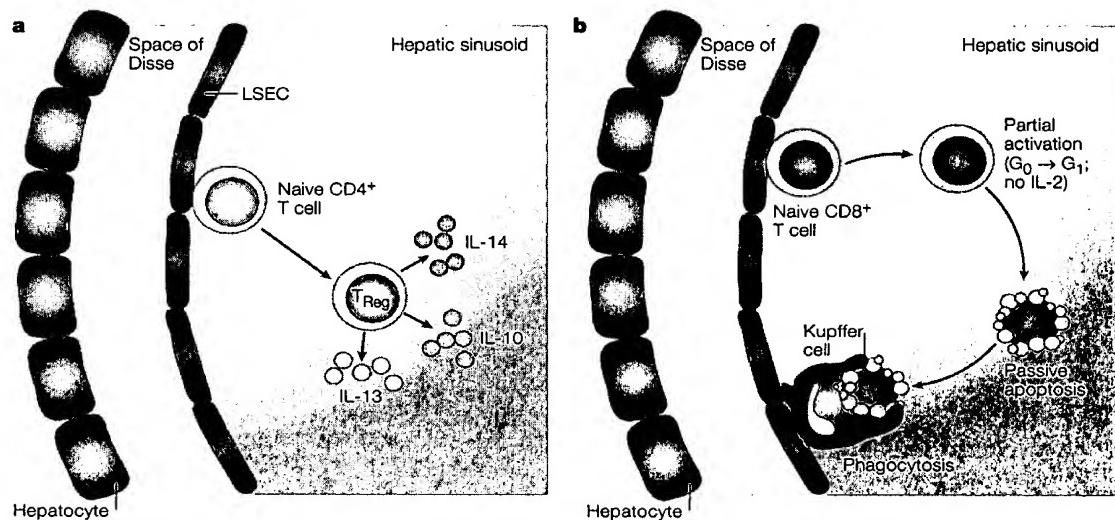


Figure 5 | Induction of T-cell tolerance by interaction of T cells with liver sinusoidal endothelial cells. **a** | Interaction of naive CD4⁺ T cells with liver sinusoidal endothelial cells (LSECs) results in differentiation of the T cells to a regulatory (T_{Reg}) phenotype. This is probably because LSECs normally produce interleukin-10 (IL-10), which favours the regulatory pathway of CD4⁺ T-cell differentiation. **b** | Interaction of naive CD8⁺ T cells with sinusoidal endothelium results in partial activation of the T cells, followed by passive cell death. The reason for this is unknown, but I propose that continuous exposure to trace amounts of intestinal lipopolysaccharide (LPS) results in the differentiation of both LSECs and liver DCs to a state that promotes different forms of T-cell tolerance in CD4⁺ and CD8⁺ T cells. The apoptotic CD8⁺ T cells are then endocytosed by Kupffer cells.

Resting memory T cells seem to be resistant to the pro-apoptotic effects of the liver. So, virus-specific memory CD8⁺ T cells are found in this site, as are TCR-transgenic memory CD4⁺ T cells specific for a protein antigen^{100,101}. For these cells, the liver might not be a killing field, but rather a hospitable neutral zone.

Immune suppression, regulation and deviation

These two mechanisms to promote CD8⁺ T-cell apoptosis are not the only tolerogenic processes that are active in the liver. Soluble antigens passing through the sinusoids are taken up by LSECs and presented to both CD8⁺ T cells and CD4⁺ T cells. T cells that are primed by LSECs might become activated and undergo proliferation, but they fail to sustain secretion of IL-2 and IFN- γ . CD8⁺ T cells that are primed by LSECs fail to differentiate into cytotoxic effector cells, whereas CD4⁺ T cells that are primed by LSECs might differentiate towards an anti-inflammatory (IL-4- and IL-10-secreting) phenotype⁹⁷ (FIG. 5a). The local synthesis of IL-10 by T cells primed by LSECs might be important, because IL-10 alters the expression of chemokine receptors by DCs in ways that would be expected to disrupt their homing to lymphoid tissues. Specifically, IL-10 causes the increased expression of CCR5 and the decreased expression of CCR7 by DCs, which renders them less responsive to the lymphoid-tissue chemokine CCL21 (REFS 122,123). The synthesis of IL-10 is prevalent in the liver. In addition to LSECs, mouse liver-derived DCs also preferentially induce the synthesis of IL-10 by CD4⁺ T cells, in contrast to bone-marrow-derived DCs, which preferentially induce IFN- γ synthesis⁹⁰. In rat liver allografts, an early phase of T-cell apoptosis is followed by the accumulation of CD4⁺CD45RC^{low} cells that produce IL-13

(REF. 124). So, although liver allografts seem to be protected in the acute phase by apoptosis of host CD8⁺ T cells, they might survive in the long term due to the presence of host CD4⁺ regulatory T cells.

What overcomes base-line tolerance?

I have described the liver as an organ in which the antigen-presenting capacity of LSECs and many DCs is biased strongly towards the induction of CD4⁺ T cells with a regulatory phenotype, whereas both CD8⁺ T cells that are activated systemically that localize to the liver, and naive CD8⁺ T cells that first encounter antigen in the liver, are predisposed strongly to undergo apoptosis. The problem is to understand how this default state of tolerance might be reversed to allow priming of T cells to occur and effector responses to be delivered. The solution is not self-evident, but there are some clues.

Type 1 interferons (IFN- α/β) activate a cytokine/chemokine cascade that recruits T cells during virus infections^{34,35}. Such IFN synthesis occurs in tissue cells, including hepatocytes, in response to virus infection. In addition, type 1 IFNs induce the synthesis of IL-15, which promotes the survival of CD8⁺ T cells^{125,126}. So, synthesis of type 1 IFNs might be an important event that overcomes liver tolerance and allows an intrahepatic T-cell response to proceed.

A second factor might be the regulation of DC function. The maturation of DC precursors seems to be a two-stage process. Initial maturation results in upregulation of the antigen-processing machinery (including MHC class I and II molecules), expression of co-stimulatory molecules (CD80 and CD86) and the synthesis of IL-10. DCs at this developmental stage prime regulatory T cells preferentially, and their

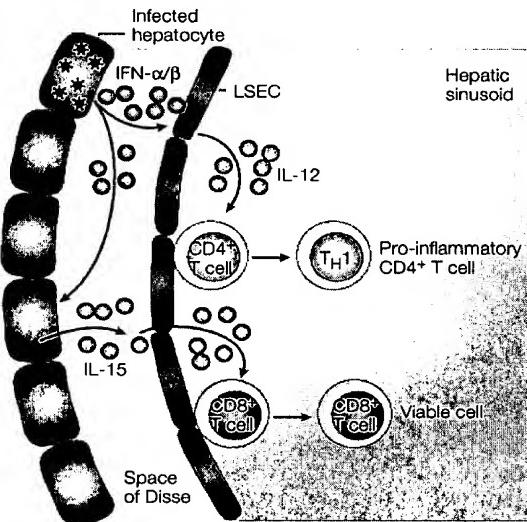


Figure 6 | An hypothesis to explain how the tolerant state of the liver is reversed, resulting in T-cell priming and immunity. This hypothesis gives type 1 interferons (IFN- α/β) a central role in the normal liver, and it proposes that chronic exposure to lipopolysaccharide results in the desensitization of sinusoidal endothelial cells to activation signals. These cells present antigen in the context of co-stimulatory molecules, but do not secrete pro-inflammatory cytokines. Therefore, CD4 $^{+}$ T cells become regulatory, whereas CD8 $^{+}$ T cells undergo passive cell death, as shown in FIG. 5. As shown here, under the influence of type 1 IFNs in an infected liver, everything changes. A virus-infected hepatocyte secretes type 1 IFNs, which act on liver sinusoidal endothelial cells (LSECs), causing them to secrete interleukin-12 (IL-12) and promote the differentiation of CD4 $^{+}$ T cells to inflammatory T helper 1 ($T_{H}1$) cells. In parallel, type 1 IFNs act on other parenchymal cells, causing them to secrete IL-15, a survival factor for CD8 $^{+}$ T cells. Not shown in this figure is the type 1 IFN-initiated cytokine/chemokine cascade that promotes T-cell recruitment (FIG. 3).

phenotype and functions are markedly similar to those of LSECs. By contrast, the final maturation of DCs results in the synthesis of IL-6, IL-12 and tumour-necrosis factor ('TNF'), and such DCs prime CD4 $^{+}$ T cells to deliver an immune response¹²⁷. The first maturation step is induced by diverse signals, including exposure to elements of the gut flora¹²⁸, whereas the second step is induced by exposure to LPS¹²⁹. I have already considered the effects of intestinal LPS on promoting the expression of adhesion molecules in the liver⁸³, and this effect indicates that LPS should also promote the final maturation of liver DCs. However, liver DCs prime CD4 $^{+}$ T cells to have a regulatory function⁹⁰. How can this be explained?

I would like to put forward the argument that tolerance-inducing liver DCs are in a state of partial LPS resistance, brought about by continuous exposure to LPS. So, they are refractory to final maturation signals through TLR4 and remain in a state that promotes the differentiation of regulatory T cells. If this is the case, a distinct signal must be required to promote final DC maturation and, thereby, the priming of T cells to deliver

effector function. This requirement is met by type 1 IFNs. In addition to its other functions, which have been discussed already, IFN- α promotes DC maturation^{130,131}, and the type 1 IFNs have been proposed to be a crucial link between innate and adaptive immunity¹³².

On the basis of this discussion, I propose the following model. First, the main reason for CD4 $^{+}$ T-cell tolerance induced by antigen presentation in the liver is that both DCs and LSECs respond to the continuous presence of LPS of intestinal origin by becoming unresponsive to TLR4 signals and by assuming a differentiation state that promotes the activation of CD4 $^{+}$ regulatory T cells. This has been shown for LSECs by Knolle *et al.*¹³³, and I propose that it applies to liver DCs also. Second, the main reason for CD8 $^{+}$ T-cell tolerance in the liver is that liver DCs and LSECs cause partial activation of CD8 $^{+}$ T cells, leading to PCD. Data from my own laboratory show an important role for LSECs in inducing CD8 $^{+}$ T-cell trapping and death²⁵. Third, pathogens that elicit a type 1 IFN response promote naive T-cell recruitment, switch the function of liver DCs and LSECs so that they promote the differentiation of CD4 $^{+}$ T cells, and allow full activation and survival of CD8 $^{+}$ T cells (FIG. 6). Thereby, an effective immune response will be delivered. Finally, it follows that pathogens that persist in the liver either suppress type 1 IFN production or evade the effects of type 1 IFNs.

FAS expression and hepatocyte damage

The location of the liver downstream from the huge absorptive surface of the gastrointestinal tract places it in the front line in relation to toxic chemicals, and the liver has evolved the capacity to detoxify potentially dangerous molecules, often through conjugation to glucuronic acid followed by excretion into the bile. However, the uptake of such molecules by hepatocytes poses a constant risk of DNA damage, which could lead to mutation and carcinogenesis. The capacity to induce rapid apoptosis of mutant hepatocytes might explain why hepatocytes express FAS on their membranes. In mice, the Fas signalling pathway is active in these cells, and they are always poised to undergo apoptosis; this can be shown by the *in vivo* injection of Fas-specific antibodies¹³⁴ or by the co-culture of hepatocytes with FasL-expressing cells¹³⁵. This capacity to undergo apoptosis is linked to the potential for extensive regeneration, which is manifested as recovery after acute hepatitis, liver regeneration after partial hepatectomy¹³⁶ and the restoration of full hepatocyte mass after the transplantation of a 'split liver'¹³⁷. The regeneration of the liver is linked closely to its resident lymphocytes. During the early stages of liver regeneration, there is a rapid, transient increase in the proportion of resident NKT cells⁶⁶. This raises the possibility that, similar to other populations of T cells with a restricted TCR repertoire^{138,139}, these cells might be involved in the response to tissue damage. However, the role of lymphocytes in liver regeneration is unclear, and NK cells have been proposed to both support¹⁴⁰ and suppress¹⁴¹ regeneration.

The expression of Fas by mouse hepatocytes places them at risk of acute damage whenever activated lymphocytes accumulate in the liver. In the simplified model of systemic CD8⁺ T-cell activation induced by the injection of antigenic peptide into TCR-transgenic mice, T-cell accumulation and apoptosis in the liver was accompanied by hepatocyte damage, resulting in histological lesions and elevation of the level of serum aminotransaminases^{30,142}. Such damage was reduced in Fas-deficient mice¹⁴³, which indicates that FasL — either on the CD8⁺ T cells themselves or induced by their presence — engages Fas on the hepatocytes. In humans, the situation is more complicated. The basal level of expression of FAS by human hepatocytes is lower than in mice, but it is elevated in inflammatory conditions, including infection with HBV or HCV¹⁴⁴. This does not seem to be associated with hepatocyte death. In fact, the expression of FAS in the infected liver might be part of antiviral defences during infection with HCV, because it is negatively correlated with liver damage, as assessed by the level of alanine aminotransaminase¹⁴⁵. FAS expression is also a good predictor of the response to IFN- α therapy¹⁴⁶. *In vivo*, the limiting factor might be the local expression of FASL. T cells could potentially provide FASL in two ways: by its expression on activated T cells^{147,148} or by an immune

interaction with hepatocytes in which the ligation of CD40 induces the expression of FASL by the hepatocytes themselves¹⁴⁹. This might be what is happening when mice are treated with a superantigen, which induces the expression of FasL on many tissues, including the liver¹²⁰.

Concluding remarks

The global prevalence of infections of the liver, including malaria, HBV and HCV, makes it important that we understand the distinctive constraints on T-cell immunity in this organ. Vaccines against malaria and HCV are still elusive, and the large number of people who are infected with liver pathogens makes therapy, as well as prevention, an important goal of immunological intervention. Antigen presentation by LSECs, and possibly by hepatocytes, might frustrate efforts to prime an effective immune response, and pro-apoptotic mechanisms might eliminate activated liver-specific CD8⁺ T cells. Highly activated T cells are dangerous in the liver, because hepatocytes express the FAS death receptor and are susceptible to immunopathology. In the face of these obstacles, it is highly desirable that we devise strategies to modify the natural history of such chronic infections by manipulating the immune system to deliver protective responses without unacceptable immunopathology.

- Wang, C. H., Tschen, S. Y., Heinrichy, U., Weber, M. & Lehming, B. Immune response to hepatitis A virus capsid proteins after infection. *J. Clin. Microbiol.* **34**, 707–713 (1996).
- Homberger, F. R. Enterotropic mouse hepatitis virus. *Lab. Anim.* **31**, 97–115 (1997).
- Lavi, E. & Wang, Q. The protective role of cytotoxic T cells and interferon against coronavirus invasion of the brain. *Adv. Exp. Med. Biol.* **380**, 145–149 (1995).
- Good, M. F. Development of immunity to malaria may not be an entirely active process. *Parasite Immunol.* **17**, 55–59 (1995).
- Rehmann, B., Ferrari, C., Pasquini, C. & Chisari, F. V. The hepatitis B virus persists for decades after patients' recovery from acute viral hepatitis despite active maintenance of a cytotoxic T-lymphocyte response. *Nature Med.* **2**, 1104–1108 (1996).
- Spengler, U., Lechmann, M., Irgang, B., Dumoulin, F. L. & Sauerbruch, T. Immune responses in hepatitis C virus infection. *J. Hepatol.* **24**, 20–25 (1996).
- Calne, R. Y. *et al.* Induction of immunological tolerance by porcine liver allografts. *Nature* **223**, 472–476 (1969).
- van der Bruggen, P. *et al.* A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science* **254**, 1643–1647 (1991).
- Braet, F., Luo, D., Spector, I., Vermijlen, D. & Wisse, E. In *The Liver Biology and Pathobiology* (eds Arias, I. *et al.*) 437–453 (Lippincott, Williams and Wilkins, Philadelphia, 2001).
- Wissem, E. In *The Reticuloendothelial System. A Comprehensive Treatise* (eds Carr, I. & Daems, W. T.) 361–377 (Plenum, New York and London, 1980).
- MacPhee, P. J., Schmidt, E. E. & Groom, A. C. Evidence for Kupffer-cell migration along liver sinusoids, from high-resolution *in vivo* microscopy. *Am. J. Physiol.* **263**, G17–G23 (1992).
- Shi, J., Fujieda, H., Kokubo, Y. & Wake, K. Apoptosis of neutrophils and their elimination by Kupffer cells in rat liver. *Hepatology* **24**, 1256–1263 (1996).
- Ruzitzu, M., Carle, E. C., Montanari, M. R., Maietta, G. & Dini, L. Modulation of cell-surface expression of liver carbohydrate receptors during *in vivo* induction of apoptosis with lead nitrate. *Cell Tissue Res.* **298**, 105–112 (1999).
- Filice, G. A. Antimicrobial properties of Kupffer cells. *Infect. Immun.* **56**, 1430–1435 (1988).
- Wardle, E. N. Kupffer cells and their function. *Liver* **7**, 63–75 (1987).
- Ruggiero, G. *et al.* Clearance of viable Calmette-Guerin bacillus by the *in vitro* isolated and perfused rat liver. *Acta Hepatoenterol. (Stuttg.)* **24**, 102–105 (1977).
- Rogoff, T. M. & Lipsky, P. E. Antigen presentation by isolated guinea-pig Kupffer cells. *J. Immunol.* **124**, 1740–1744 (1980).
- Gregory, S. H. & Wing, E. J. Accessory function of Kupffer cells in the antigen-specific blastogenic response of an L3T4⁺ T-lymphocyte clone to *Listeria monocytogenes*. *Infect. Immun.* **58**, 2313–2319 (1990).
- Roland, C. R., Walp, L., Stack, R. M. & Flye, M. W. Outcome of Kupffer-cell antigen presentation to a cloned murine T_H1 lymphocyte depends on the inducibility of nitric oxide synthase by IFN- γ . *J. Immunol.* **153**, 5453–5464 (1994).
- Yu, S., Nakafusa, Y. & Flye, M. W. Portal-vein administration of donor cells promotes peripheral allospecific hyporesponsiveness and graft tolerance. *Surgery* **116**, 229–234 (1994).
- Roland, C. R., Mangino, M. J., Duffy, B. F. & Flye, M. W. Lymphocyte suppression by Kupffer cells prevents portal venous tolerance induction: a study of macrophage function after intravenous gadolinium. *Transplantation* **55**, 1151–1158 (1993).
- Norris, S. *et al.* Resident human hepatic lymphocytes are phenotypically different from circulating lymphocytes. *J. Hepatol.* **28**, 84–90 (1998).
- Crispe, I. N. & Mehal, W. Z. Strange brew: T cells in the liver. *Immunol. Today* **17**, 522–525 (1996).
- Pruvot, F. R. *et al.* Characterization, quantification and localization of passenger T lymphocytes and NK cells in human liver before transplantation. *Transpl. Int.* **8**, 273–279 (1995).
- Mehal, W. Z., Juedes, A. E. & Crispe, I. N. Selective retention of activated CD8⁺ T cells by the normal liver. *J. Immunol.* **163**, 3202–3210 (1999).
- Klugewitz, K. *et al.* Differentiation-dependent and subset-specific recruitment of T-helper cells into murine liver. *Hepatology* **35**, 568–578 (2002).
- Klugewitz, K. *et al.* Immunomodulatory effects of the liver: deletion of activated CD4⁺ effector cells and suppression of IFN- γ -producing cells after intravenous protein immunotherapy. *J. Immunol.* **169**, 2407–2413 (2002).
- Huang, L., Sye, K. & Crispe, I. N. Proliferation and apoptosis of abundant B220 CD4⁺ CD8⁺ T cells in the normal mouse liver. *Int. Immunol.* **6**, 533–540 (1994).
- Rennó, T., Hahne, M., Tschoopp, J. & MacDonald, H. R. Peripheral T cells undergoing superantigen-induced apoptosis *in vivo* express B220 and upregulate Fas and Fas ligand. *J. Exp. Med.* **183**, 431–437 (1996).
- Huang, L., Soldevila, G., Leeker, M., Flavell, R. & Crispe, I. N. The liver eliminates T cells undergoing antigen-triggered apoptosis *in vivo*. *Immunity* **1**, 741–749 (1994). **In this study, my colleagues and I showed that peripheral deletion of CD8⁺ T cells is accompanied by liver lymphocytosis, intrahepatic T-cell apoptosis and hepatocyte damage.**
- Bouwens, L., Remets, L., Baekeland, M., Van Bossuyt, H. & Wissem, E. Large granular lymphocytes or 'pit cells' from rat liver: isolation, ultrastructural characterization and natural killer activity. *Eur. J. Immunol.* **17**, 37–42 (1987).
- McIntyre, K. W. & Welsh, R. M. Accumulation of natural killer and cytotoxic T large granular lymphocytes in the liver during virus infection. *J. Exp. Med.* **164**, 1667–1681 (1986).
- Toyabe, S. *et al.* Requirement of IL-4 and liver NK1⁺ T cells for concanavalin-A-induced hepatic injury in mice. *J. Immunol.* **159**, 1537–1542 (1997).
- Liu, Z. X., Govindarajan, S., Okamoto, S. & Dennert, G. NK cells cause liver injury and facilitate the induction of T-cell-mediated immunity to a viral liver infection. *J. Immunol.* **164**, 6480–6486 (2000).
- Salazar-Mather, T. P., Orange, J. S. & Biron, C. A. Early murine cytomegalovirus (MCMV) infection induces liver natural killer (NK)-cell inflammation and protection through macrophage inflammatory protein 1 α (MIP-1 α)-dependent pathways. *J. Exp. Med.* **187**, 1–14 (1998).
- Itoh, Y. *et al.* Time-course profile and cell-type-specific production of monokine induced by interferon- γ in concanavalin-A-induced hepatic injury in mice: comparative study with interferon-inducible protein-10. *Scand. J. Gastroenterol.* **36**, 1344–1351 (2001).
- Watanabe, H. *et al.* Characterization of intermediate TCR cells in the liver of mice with respect to their unique IL-2R expression. *Cell. Immunol.* **149**, 331–342 (1993).
- MacDonald, H. R. Development and selection of NKT cells. *Curr. Opin. Immunol.* **14**, 250–254 (2002).
- MacDonald, H. R. Va14 NKT cells — a novel lymphoid cell lineage. *Jpn. J. Cancer Res.* **88**, inside front cover (1997).
- Eberl, G. *et al.* Tissue-specific segregation of CD1d-dependent and CD1d-independent NKT cells. *J. Immunol.* **162**, 6410–6419 (1999).
- Baron, J. L. *et al.* Activation of a nonclassical NKT-cell subset in a transgenic mouse model of hepatitis B virus infection. *Immunity* **16**, 583–594 (2002).
- Bernlagha, K., Kyri, T., Beavis, A., Teyton, L. & Bendelac, A. A thymic precursor to the NKT-cell lineage. *Science* **296**, 553–555 (2002).

43. Sato, K. *et al.* Evidence for extrathymic generation of intermediate T-cell receptor cells in the liver revealed in thymectomized, irradiated mice subjected to bone-marrow transplantation. *J. Exp. Med.* **182**, 759–767 (1995).
44. Slifka, M. K., Paganin, R. R. & Whitton, J. L. NK markers are expressed on a high percentage of virus-specific CD8⁺ and CD4⁺ T cells. *J. Immunol.* **164**, 2009–2015 (2000).
45. McMahon, C. W. *et al.* Viral and bacterial infections induce expression of multiple NK-cell receptors in responding CD8⁺ T cells. *J. Immunol.* **169**, 1444–1452 (2002).
46. Vicari, A. P., Mocci, S., Openshaw, P., O'Garra, A. & Zlotnik, A. Mouse $\gamma\delta$ TCR-NK1.1⁺ thymocytes specifically produce interleukin-4, are major histocompatibility complex class I independent, and are developmentally related to $\alpha\beta$ TCR-NK1.1⁺ thymocytes. *Eur. J. Immunol.* **26**, 1424–1429 (1996).
47. Mars, L. T. *et al.* Cutting edge: $\text{V}\alpha 14\text{-J}\alpha 28.1$ NKT cells naturally regulate experimental autoimmune encephalomyelitis in nonobese diabetic mice. *J. Immunol.* **168**, 6007–6011 (2002).
48. Laloux, V., Beaudoin, L., Jeske, D., Carnaud, C. & Lehuen, A. NKT-cell-induced protection against diabetes in $\text{V}\alpha 14\text{-J}\alpha 28.1$ transgenic nonobese diabetic mice is associated with a T_{1,2} shift circumscribed regionally to the islets and functionally to islet autoantigen. *J. Immunol.* **166**, 3749–3756 (2001).
49. Behar, S. M., Dascher, C. C., Grusby, M. J., Wang, C. R. & Brenner, M. B. Susceptibility of mice deficient in CD1D or TAP1 to infection with *Mycobacterium tuberculosis*. *J. Exp. Med.* **189**, 1973–1980 (1999).
50. Kumar, H., Belperron, A., Barthold, S. W. & Bockenstedt, L. K. Cutting edge: CD1d deficiency impairs murine host defense against the spirochete, *Borrelia burgdorferi*. *J. Immunol.* **165**, 4797–4801 (2000).
51. Duthie, M. S. & Kahn, S. J. Treatment with α -galactosylceramide fails to prevent *Trypanosoma cruzi* infection provides protection or induces failure to thrive. *J. Immunol.* **168**, 5778–5785 (2002).
52. Duthie, M. S. *et al.* During *Trypanosoma cruzi* infection CD1d-restricted NKT cells limit parasitemia and augment the antibody response to a glycoprophosphoinositol-modified surface protein. *Infect. Immun.* **70**, 36–48 (2002).
53. Romero, J. F., Eberl, G., MacDonald, H. R. & Corradini, G. CD1d-restricted NK T cells are dispensable for specific antibody responses and protective immunity against liver-stage malaria infection in mice. *Parasite Immunol.* **23**, 267–269 (2001).
54. Dao, T. *et al.* Involvement of CD1 in peripheral deletion of T lymphocytes is independent of NK T cells. *J. Immunol.* **166**, 3090–3097 (2001).
55. Cui, J. *et al.* Requirement for $\text{V}\alpha 14$ NKT cells in IL-12-mediated rejection of tumors. *Science* **278**, 1623–1626 (1997).
56. Crowe, N. Y., Smyth, M. J. & Godfrey, D. I. A critical role for natural killer T cells in immunosurveillance of methylcholanthrene-induced sarcomas. *J. Exp. Med.* **196**, 119–127 (2002).
57. Matsuda, J. L. *et al.* Tracking the response of natural killer T cells to a glycolipid antigen using CD1d tetramers. *J. Exp. Med.* **192**, 741–754 (2000).
58. Toura, I. *et al.* Cutting edge: inhibition of experimental tumor metastasis by dendritic cells pulsed with α -galactosylceramide. *J. Immunol.* **163**, 2387–2391 (1999).
59. Gonzalez-Aseguinolaza, G. *et al.* α -galactosylceramide-activated $\text{V}\alpha 14$ natural killer T cells mediate protection against murine malaria. *Proc. Natl Acad. Sci. USA* **97**, 8461–8466 (2000).
60. Kakimi, K., Guidotti, L. G., Koezuka, Y. & Chisari, F. V. Natural killer T-cell activation inhibits hepatitis B virus replication in vivo. *J. Exp. Med.* **192**, 921–930 (2000).
61. Norris, S. *et al.* Natural T cells in the human liver: cytotoxic lymphocytes with dual T-cell and natural-killer-cell phenotype and function are phenotypically heterogeneous and include $\text{V}\alpha 24\text{-J}\alpha 2$ and $\gamma\delta$ T-cell receptor-bearing cells. *Hum. Immunol.* **60**, 20–31 (1999).
62. Collins, C. *et al.* RAG1, RAG2 and pre-T-cell receptor α -chain expression by adult human hepatic T cells: evidence for extrathymic T-cell maturation. *Eur. J. Immunol.* **26**, 3114–3118 (1996).
63. Crosbie, O. M. *et al.* In vitro evidence for the presence of hematopoietic stem cells in the adult human liver. *Hepatology* **29**, 1193–1198 (1999).
64. Matsui, K. *et al.* *Propionibacterium acnes* treatment diminishes CD4⁺NK1.1⁺ T cells but induces type I T cells in the liver by induction of IL-12 and IL-18 production from Kupffer cells. *J. Immunol.* **159**, 97–106 (1997).
65. Guebre-Xavier, M. *et al.* Altered hepatic lymphocyte subpopulations in obesity-related murine fatty livers: potential mechanism for sensitization to liver damage. *Hepatology* **31**, 633–640 (2000).
66. Minagawa, M. *et al.* Intensive expansion of natural killer T cells in the early phase of hepatocyte regeneration after partial hepatectomy in mice and its association with sympathetic nerve activation. *Hepatology* **31**, 907–915 (2000).
67. Gossman, J., Lohler, J., Utermohlen, O. & Lehmann-Grube, F. Murine hepatitis caused by lymphocytic choriomeningitis virus. II. Cells involved in pathogenesis. *Lab. Invest.* **72**, 559–570 (1995).
68. Kakumu, S. *et al.* Comparisons of peripheral blood and hepatic lymphocyte subpopulations and interferon production in chronic viral hepatitis. *J. Clin. Lab. Immunol.* **33**, 1–6 (1990).
69. Pham, B. N. *et al.* Flow cytometry CD4⁺/CD8⁺ ratio of liver-derived lymphocytes correlates with viral replication in chronic hepatitis B. *Clin. Exp. Immunol.* **97**, 403–410 (1994).
70. Fiore, G. *et al.* CD45RA and CD45RO isoform expression on intrahepatic T-lymphocytes in chronic hepatitis C. *Microbiol.* **92**, 73–82 (1997).
71. Tseng, C. T., Miskovsky, E., Houghton, M. & Klimpel, G. R. Characterization of liver T-cell receptor $\gamma\delta$ T cells obtained from individuals chronically infected with hepatitis C virus (HCV): evidence for these T cells playing a role in the liver pathology associated with HCV infections. *Hepatology* **33**, 1312–1320 (2001).
72. Crispe, I. N., Dao, T., Klugewitz, K., Mehal, W. Z. & Motz, D. P. The liver as a site of T-cell apoptosis: graveyard or killing field? *Immunol. Rev.* **174**, 47–62 (2000).
73. Volpes, R., van den Cord, J. J. & Desmet, V. J. Hepatic expression of intercellular adhesion molecule-1 (ICAM-1) in viral hepatitis. *B. Hepatology* **12**, 148–154 (1990).
74. Garcia-Monzon, C. *et al.* Vascular adhesion molecule expression in viral chronic hepatitis: evidence of neangiogenesis portal tracts. *Gastroenterology* **108**, 231–241 (1995).
75. Mochizuki, K. *et al.* B7/BB-1 expression and hepatitis activity in liver tissues of patients with chronic hepatitis C. *Hepatology* **25**, 713–718 (1997).
76. Hiramatsu, N. *et al.* Immunohistochemical detection of Fas antigen in liver tissue of patients with chronic hepatitis C. *Hepatology* **19**, 1354–1359 (1994).
77. Butcher, E. C., Williams, M., Youngman, K., Rott, L. & Briskin, M. Lymphocyte trafficking and regional immunity. *Adv. Immunol.* **72**, 209–253 (1999).
78. Wong, J. *et al.* A minimal role for selectins in the recruitment of leukocytes into the inflamed liver microvasculature. *J. Clin. Invest.* **99**, 2782–2790 (1997).
79. Iigo, Y. *et al.* Constitutive expression of ICAM-1 in rat microvascular systems analyzed by laser confocal microscopy. *Am. J. Physiol.* **273**, H138–H147 (1997).
80. McNab, G. *et al.* Vascular adhesion protein 1 mediates binding of T cells to human hepatic endothelium. *Gastroenterology* **110**, 522–528 (1996).
81. Harju, K., Glumoff, V. & Hallman, M. Ontogeny of Toll-like receptors Tlr2 and Tlr4 in mice. *Pediatr. Res.* **49**, 81–83 (2001).
82. Faure, E. *et al.* Bacterial lipopolysaccharide and IFN- γ induce Toll-like receptor 2 and Toll-like receptor 4 expression in human endothelial cells: role of NF- κ B activation. *J. Immunol.* **168**, 2018–2024 (2001).
83. van Oosten, M., van de Bilt, E., de Vries, H. E., van Berkel, T. J. & Kuiper, J. Vascular adhesion molecule-1 and intercellular adhesion molecule-1 expression on rat liver cells after lipopolysaccharide administration *in vivo*. *Hepatology* **22**, 1538–1546 (1995).
84. Matsuno, K., Ezaki, T., Kudo, S. & Uehara, Y. A life stage of particle-laden rat dendritic cells *in vivo*: their terminal division, active phagocytosis and translocation from the liver to the draining lymph. *J. Exp. Med.* **183**, 1865–1878 (1996).
85. Dieu, M. C. *et al.* Selective recruitment of immature and mature dendritic cells by distinct chemokines expressed in different anatomic sites. *J. Exp. Med.* **188**, 373–386 (1998).
86. Saeki, H., Moore, A. M., Brown, M. J. & Hwang, S. T. Cutting edge: secondary lymphoid-tissue chemokine (SLC) and CC-chemokine receptor 7 (CCR7) participate in the emigration pathway of mature dendritic cells from the skin to regional lymph nodes. *J. Immunol.* **162**, 2472–2475 (1999).
87. Yoneyama, H. *et al.* Regulation by chemokines of circulating dendritic-cell precursors, and the formation of portal tract-associated lymphoid tissue, in a granulomatous liver disease. *J. Exp. Med.* **183**, 35–49 (2001).
88. Prickett, T. C., McKenzie, J. L. & Hart, D. N. Characterization of interstitial dendritic cells in human liver. *Transplantation* **46**, 754–761 (1988).
89. Traver, D. *et al.* Development of CD8 α -positive dendritic cells from a common myeloid progenitor. *Science* **290**, 2152–2154 (2000).
90. O'Connell, P. J., Morelli, A. E., Logar, A. J. & Thomson, A. W. Phenotypic and functional characterization of mouse hepatic CD8 α lymphoid-related dendritic cells. *J. Immunol.* **165**, 795–803 (2000).
91. Uwatoku, R. *et al.* Kupffer-cell-mediated recruitment of rat dendritic cells to the liver: roles of N-acetylgalactosamine-specific sugar receptors. *Gastroenterology* **121**, 1460–1472 (2001).
92. Uwatoku, R. *et al.* Asialoglycoprotein receptors on rat dendritic cells: possible roles for binding with Kupffer cells and ingesting virus particles. *Arch. Histol. Cytol.* **64**, 223–232 (2001).
93. Higashi, N. *et al.* The macrophage C-type lectin specific for galactose/N-acetylgalactosamine is an endocytic receptor expressed on monocyte-derived immature dendritic cells. *J. Biol. Chem.* **277**, 20686–20693 (2002).
94. Albert, M. L., Sauter, B. & Bhardwaj, N. Dendritic cells acquire antigen from apoptotic cells and induce class-I-restricted CTLs. *Nature* **392**, 86–89 (1998).
95. Albert, M. L. *et al.* Immature dendritic cells phagocytose apoptotic cells via $\alpha\beta$ ₂ and CD36, and cross-present antigens to cytotoxic T lymphocytes. *J. Exp. Med.* **188**, 1359–1368 (1998).
96. Lohse, A. W. *et al.* Antigen-presenting function and B7 expression of murine sinusoidal endothelial cells and Kupffer cells. *Gastroenterology* **110**, 1175–1181 (1996).
97. Knolle, P. A. *et al.* Induction of cytokine production in naïve CD4⁺ T cells by antigen-presenting murine liver sinusoidal endothelial cells but failure to induce differentiation toward T₁ T cells. *Gastroenterology* **116**, 1428–1440 (1999).
98. Limmer, A. *et al.* Efficient presentation of exogenous antigen by liver endothelial cells to CD8⁺ T cells results in antigen-specific T-cell tolerance. *Nature Med.* **6**, 1348–1354 (2000). **In this paper, Knolle and colleagues describe the unique properties of liver sinusoidal endothelial cells as antigen-presenting cells that promote tolerance.**
99. Leifeld, L. *et al.* Enhanced expression of CD80 (B7-1), CD86 (B7-2) and CD40 and their ligands CD28 and CD154 in fulminant hepatic failure. *Am. J. Pathol.* **154**, 1711–1720 (1999).
100. Masopust, D., Veys, V., Marzo, A. L. & Lefrancois, L. Preferential localization of effector memory cells in nonlymphoid tissue. *Science* **291**, 2413–2417 (2001).
101. Reinhardt, R. L., Khoruts, A., Merica, R., Zell, T. & Jenkins, M. K. Visualizing the generation of memory CD4 T cells in the whole body. *Nature* **410**, 101–105 (2001).
102. Ando, K., Guidotti, L. G., Cerny, A., Ishikawa, T. & Chisari, F. V. CTL access to tissue antigen is restricted *in vivo*. *J. Immunol.* **153**, 482–488 (1994).
103. Bertolini, P., Bowen, D. G., McCaughan, G. W. & Fazekas de St Groth, B. Antigen-specific primary activation of CD8⁺ T cells within the liver. *J. Immunol.* **168**, 5430–5438 (2001).
104. Bertolini, P., Trescol-Biemont, M. C. & Rabourdin-Combe, C. Hepatocytes induce functional activation of naïve CD8⁺ T lymphocytes but fail to promote survival. *Eur. J. Immunol.* **28**, 221–236 (1998). **One explanation for liver tolerance is that antigen presentation by non-professional antigen-presenting cells, such as hepatocytes, causes T-cell apoptosis. This paper describes experiments in favour of this theory.**
105. Bertolini, P. *et al.* Death by neglect as a deleterious mechanism of peripheral tolerance. *Int. Immunol.* **11**, 1225–1238 (1999).
106. Qian, S. *et al.* Apoptosis within spontaneously accepted mouse liver allografts: evidence for deletion of cytotoxic T cells and implications for tolerance induction. *J. Immunol.* **158**, 4654–4661 (1997). **Qian and colleagues show that apoptosis is an important feature of the lymphocyte influx to a liver allograft.**
107. Meyer, D. *et al.* Apoptosis of alloreactive T cells in liver allografts during tolerance induction. *Transplant. Proc.* **31**, 474 (1999).
108. Lohman, B. L., Razi, E. S. & Welsh, R. M. T-lymphocyte downregulation after acute viral infection is not dependent on CD95 (Fas) receptor-ligand interactions. *J. Virol.* **70**, 8199–8203 (1996).
109. Leonardo, M. *et al.* Mature T lymphocyte apoptosis – immune regulation in a dynamic and unpredictable antigenic environment. *Annu. Rev. Immunol.* **17**, 221–253 (1999).
110. Rathmell, J. C. & Thompson, C. B. The central effectors of cell death in the immune system. *Annu. Rev. Immunol.* **17**, 781–828 (1999).
111. Slep, E. A., Adrain, C. & Martin, S. J. Serial killers: ordering caspase activation events in apoptosis. *Cell Death Differ.* **6**, 1067–1074 (1999).
112. Leonardo, M. J. Interleukin-2 programs mouse $\alpha\beta$ T lymphocytes for apoptosis. *Nature* **353**, 858–861 (1991).
113. Refaeli, Y., Van Paris, L., London, C. A., Tschopp, J. & Abbas, A. K. Biochemical mechanisms of IL-2-regulated Fas-mediated T-cell apoptosis. *Immunity* **8**, 615–623 (1998).

114. Van Paris, L., Refaeli, Y., Abbas, A. K. & Baltimore, D. Autoimmunity as a consequence of retrovirus-mediated expression of c-FLIP in lymphocytes. *Immunity* **11**, 763–770 (1999).
115. Medema, J. P. et al. FLICE is activated by association with the CD95 death-inducing signaling complex (DISC). *EMBO J.* **16**, 2794–2804 (1997).
116. Lee, Y. C. et al. Hepatocytes and liver nonparenchymal cells induce apoptosis in activated T cells. *Transplant. Proc.* **31**, 784 (1999).
117. DeSilva, D. R., Urdahl, K. B. & Jenkins, M. K. Clonal anergy is induced *in vitro* by T-cell receptor occupancy in the absence of proliferation. *J. Immunol.* **147**, 3261–3267 (1991).
118. Li, W. et al. Costimulation blockade promotes the apoptotic death of graft-infiltrating T cells and prolongs survival of hepatic allografts from FLT3L-treated donors. *Transplantation* **72**, 1423–1432 (2001).
119. Liu, Z. X., Govindarajan, S., Okamoto, S. & Dennert, G. Fas-mediated apoptosis causes elimination of virus-specific cytotoxic T cells in the virus-infected liver. *J. Immunol.* **166**, 3035–3041 (2001).
120. Bonfoco, E. et al. Inducible nonlymphoid expression of Fas ligand is responsible for superantigen-induced peripheral deletion of T cells. *Immunity* **9**, 711–720 (1998).
121. Badovinac, V. P., Twinner, A. R. & Harty, J. T. Regulation of antigen-specific CD8+ T-cell homeostasis by perforin and interferon- γ . *Science* **290**, 1354–1358 (2000).
122. D'Amico, G. et al. Uncoupling of inflammatory chemokine receptors by IL-10: generation of functional decoys. *Nature Immunol.* **1**, 387–391 (2000).
123. Takeyama, T. et al. Mammalian and viral IL-10 enhance CC-chemokine receptor 5 but down-regulate CC-chemokine receptor 7 expression by myeloid dendritic cells: impact on chemotactic responses and *in vivo* homing ability. *J. Immunol.* **166**, 7136–7143 (2001).
124. Otto, C. et al. Mechanisms of tolerance induction after rat liver transplantation: intrahepatic CD4+ T cells produce different cytokines during rejection and tolerance in response to stimulation. *J. Gastrointest. Surg.* **6**, 455–463 (2002).
125. Zhang, X., Sun, S., Hwang, I., Tough, D. F. & Sprent, J. Potent and selective stimulation of memory-phenotype CD8+ T cells *in vivo* by IL-15. *Immunity* **8**, 591–599 (1998).
126. Mattei, F., Schiavoni, G., Belardelli, F. & Tough, D. F. IL-15 is expressed by dendrite cells in response to type I IFN, double-stranded RNA or lipopolysaccharide and promotes dendrite-cell activation. *J. Immunol.* **167**, 1179–1187 (2001).
127. Lutz, M. & Schuler, G. Immature, semi-mature and fully mature dendrite cells: which signals induce tolerance or immunity? *Trends Immunol.* **23**, 445–449 (2002).
128. Christensen, H. R., Frokjaer, H. & Pestka, J. J. Lactobacilli differentially modulate expression of cytokines and maturation surface markers in murine dendrite cells. *J. Immunol.* **168**, 171–178 (2002).
129. Granucci, F., Vizzardi, C., Virzì, E., Rescigno, M. & Ricciardi-Castagnoli, P. Transcriptional reprogramming of dendrite cells by differentiation stimuli. *Eur. J. Immunol.* **31**, 2539–2546 (2001).
130. Luft, T. et al. Type I IFNs enhance the terminal differentiation of dendrite cells. *J. Immunol.* **161**, 1947–1953 (1998).
131. Radvanyi, L. G., Banerjee, A., Weir, M. & Messner, H. Low levels of interferon- α induce CD86 (B7.2) expression and accelerate dendrite-cell maturation from human peripheral-blood mononuclear cells. *Scand. J. Immunol.* **50**, 499–509 (1999).
132. Kadewald, N., Antonenko, S., Lau, J. Y. & Liu, Y. J. Natural interferon- α/β -producing cells link innate and adaptive immunity. *J. Exp. Med.* **192**, 219–226 (2000).
133. Knolle, P. A. et al. Endotoxin down-regulates T-cell activation by antigen-presenting liver sinusoidal endothelial cells. *J. Immunol.* **162**, 1401–1407 (1999).
134. Ogasawara, J. et al. Lethal effects of the anti-Fas antibody in mice. *Nature* **368**, 806–809 (1993).
135. Schlosser, S. F. et al. Induction of murine hepatocyte death by membrane-bound CD95 (Fas/APO-1)-ligand: characterization of an *in vitro* system. *Hepatology* **32**, 779–785 (2000).
136. Kountouras, J., Boura, P. & Lygidakis, N. J. Liver regeneration after hepatectomy. *Hepatogastroenterology* **48**, 556–562 (2001).
137. Malago, M., Rogiers, X. & Broelsch, C. E. Reduced-size hepatic allografts. *Annu. Rev. Med.* **46**, 507–512 (1995).
138. Roberts, S. J. et al. T-cell $\alpha\beta$ - and $\gamma\delta$ -deficient mice display abnormal but distinct phenotypes toward a natural, widespread infection of the intestinal epithelium. *Proc. Natl. Acad. Sci. USA* **93**, 11774–11779 (1996).
139. Boisneau, R. & Havran, W. Modulation of epithelial-cell growth by intraepithelial $\gamma\delta$ T cells. *Science* **268**, 1253–1255 (1994).
140. Tamura, F. et al. FK506 promotes liver regeneration by suppressing natural killer cell activity. *J. Gastroenterol. Hepatol.* **13**, 703–708 (1998).
141. Polimeno, L. et al. Molecular mechanisms of augmentation of liver regeneration as immunoregulator: its effect on interferon- γ expression in rat liver. *Dig. Liver Dis.* **32**, 217–225 (2000).
142. Russell, J. Q. et al. Liver damage preferentially results from CD8+ T cells triggered by high-affinity peptide antigens. *J. Exp. Med.* **188**, 1147–1157 (1998).
143. Kennedy, N. J., Russell, J. Q., Michail, N. & Budd, R. C. Liver damage by infiltrating CD8+ T cells is Fas dependent. *J. Immunol.* **167**, 6654–6662 (2001).
144. Hayashi, N. & Mita, E. Involvement of Fas system-mediated apoptosis in pathogenesis of viral hepatitis. *J. Viral Hepat.* **6**, 357–365 (1999).
145. Fiore, G. et al. Liver tissue expression of CD80 and CD95 antigens in chronic hepatitis C: relationship with biological and histological disease activities. *Microbiol.* **97**, 29–38 (1999).
146. Abe, S., Kotoh, K., Arao, S., Tabaru, A. & Otsuki, M. Fas antigen expression on hepatocytes predicts the short- and long-term response to interferon therapy in patients with chronic hepatitis C. *Scand. J. Gastroenterol.* **36**, 326–331 (2001).
147. Kondo, T., Suda, T., Fukuyama, H., Adachi, M. & Nagata, S. Essential roles of the Fas ligand in the development of hepatitis. *Nature Med.* **3**, 409–413 (1997).
148. Bobe, P. et al. Fas-mediated liver damage in MRL hemopoietic chimeras undergoing lpr-matched graft-versus-host disease. *J. Immunol.* **159**, 4197–4204 (1997).
149. Afford, S. C. et al. CD40 activation induces apoptosis in cultured human hepatocytes via induction of cell-surface Fas-ligand expression and amplifies Fas-mediated hepatocyte death during allograft rejection. *J. Exp. Med.* **189**, 441–446 (1999).
150. Alter, H. J. & Seeff, L. B. Recovery, persistence and sequelae in hepatitis C virus infection: a perspective on long-term outcome. *Semin. Liver Dis.* **20**, 17–35 (2000).
151. Wong, D. K. et al. Liver-derived CTL in hepatitis C virus infection: breadth and specificity of responses in a cohort of persons with chronic infection. *J. Immunol.* **160**, 1479–1488 (1998).
152. Lechner, F. et al. CD8+ T-lymphocyte responses are induced during acute hepatitis C virus infection but are not sustained. *Eur. J. Immunol.* **30**, 2479–2487 (2000).
153. Lechner, F. et al. Analysis of successful immune responses in persons infected with hepatitis C virus. *J. Exp. Med.* **191**, 1499–1512 (2000).
154. Cooper, S. et al. Analysis of a successful immune response against hepatitis C virus. *Immunity* **10**, 439–449 (1999). **This paper shows the importance of the magnitude and diversity of the cytotoxic T-lymphocyte response in determining the outcome of hepatitis C virus infection.**
155. Weiner, A. et al. Persistent hepatitis C virus infection in a chimpanzee is associated with emergence of a cytotoxic T-lymphocyte escape variant. *Proc. Natl. Acad. Sci. USA* **92**, 2755–2759 (1995).
156. Erickson, A. L. et al. The outcome of hepatitis C virus infection is predicted by escape mutations in epitopes targeted by cytotoxic T lymphocytes. *Immunity* **15**, 883–895 (2001).
157. Chang, K. M. et al. Immunological significance of cytotoxic T-lymphocyte epitope variants in patients chronically infected by the hepatitis C virus. *J. Clin. Invest.* **100**, 2376–2385 (1997).
158. Frasca, L. et al. Hypervariable region 1 variants act as TCR antagonists for hepatitis C virus-specific CD4+ T cells. *J. Immunol.* **163**, 650–658 (1999).
159. Gruner, N. H. et al. Sustained dysfunction of antiviral CD8+ T-lymphocytes after infection with hepatitis C virus. *J. Virol.* **75**, 5550–5558 (2001).
160. Kittlesen, D. J., Chianese-Bullock, K. A., Yao, Z. Q., Branciale, T. J. & Hahn, Y. S. Interaction between complement receptor gC1qR and hepatitis C virus core protein inhibits T-lymphocyte proliferation. *J. Clin. Invest.* **106**, 1239–1249 (2000).
161. Yao, Z. Q., Nguyen, D. T., Hiotellis, A. I. & Hahn, Y. S. Hepatitis C virus core protein inhibits human T-lymphocyte responses by a complement-dependent regulatory pathway. *J. Immunol.* **167**, 5264–5272 (2001).
162. Hahn, C. S., Cho, Y. G., Kang, B. S., Lester, I. M. & Hahn, Y. S. The HCV core protein acts as a positive regulator of Fas-mediated apoptosis in a human lymphoblastoid T-cell line. *Virology* **278**, 127–137 (2000).
163. Crotta, S. et al. Inhibition of natural killer cells through engagement of CD81 by the major hepatitis C virus envelope protein. *J. Exp. Med.* **195**, 35–42 (2002).
164. Sun, J. et al. Hepatitis C virus core and envelope proteins do not suppress the host's ability to clear a hepatic viral infection. *J. Virol.* **75**, 11992–11998 (2001).
165. Tiegs, G., Hentschel, J. & Wendel, A. T-cell-dependent experimental liver injury in mice inducible by concanavalin A. *J. Clin. Invest.* **90**, 196–203 (1992).
166. Tiegs, G. Experimental hepatitis and role of cytokines. *Acta Gastroenterol. Belg.* **60**, 176–179 (1997).
167. Bertolino, P., Heath, W. R., Hardy, C. L., Morahan, G. & Miller, J. F. Peripheral deletion of autoreactive CD8+ T cells in transgenic mice expressing H-2Kb in the liver. *Eur. J. Immunol.* **25**, 1932–1942 (1995).
168. Ando, K. et al. Mechanisms of class-I-restricted immunopathology. A transgenic mouse model of fulminant hepatitis. *J. Exp. Med.* **178**, 1541–1554 (1993).
169. Nakamoto, Y., Guidotti, L. G., Pasquetto, V., Schreiber, R. D. & Chisari, F. V. Differential target-cell sensitivity to CTL-activated death pathways in hepatitis B virus transgenic mice. *J. Immunol.* **158**, 5692–5697 (1997).

Acknowledgements

I would like to thank A. Livingstone, P. Knolle and R. Pierce for discussions and for constructive criticism of the manuscript, and the National Institutes of Health for research support.

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 APAF1 | B220 | caspase-8 | caspase-9 | CCL3 | CCL21 | CCR1 | CCR5 | CCR7 | CD1d | CD2 | CD7 | CD8a | CD11C | CD25 | CD40 | CD56 | CD69 | CD80 | CD86 | CD95 | CTLA4 | CXCL9 | FLIP | ICAM1 | ICAM2 | IFN- α/β | IFN- γ | IL-2 | IL-2R β | IL-4 | IL-6 | IL-10 | IL-12 | IL-15 | LFA1 | NK1.1 | RAG1 | RAG2 | TLR4 | TNF | TNFR1 | VAP1 | VCAM1

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Fetal gene transfer

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Current Opinion In Molecular Therapeutics 2007 9(5):432-438
© The Thomson Corporation ISSN 1464-8431

Gene transfer early in development for the treatment of monogenetic and other diseases could overcome major obstacles of intervention in the mature individual. Early gene transfer may prevent the onset of irreversible pathological changes, predispose the individual to immunological tolerance to the introduced protein, take advantage of the high vector to cell ratio, and provide unique access to stem cell/progenitor compartments. The past few years have witnessed the publication of five studies showing long-term correction of monogenetic disorders by fetal gene transfer. Many others have examined the use of new vector systems with therapeutic transgenes, tested their potential for treating diseases in a wide range of organs (including the brain, lung and skin), and examined the hazards of fetal application. This review gives a comprehensive summary of the development of fetal gene transfer over the past few years.

Keywords Adeno-associated virus, adenovirus, fetal gene therapy, *in utero* gene transfer, lentivirus

Introduction

In 1984, Jaenisch and coworkers explored the potential use of retroviral vectors to deliver transgenes to fetal animal tissue. They injected mid-gestation mouse embryos with a cell line engineered to produce retrovirus carrying a marker gene [1]. Expression of the marker gene was detected in various tissues of adult mice, albeit in low amounts. However, these experiments were not conducted with a therapeutic goal and it took another five years for researchers at the NIH to successfully performed fetal gene therapy. After transducing fetal sheep hematopoietic cells *ex vivo* with a retroviral vector carrying a marker gene and

re-infusing these cells into the fetus, transgene expression was detected for over a four-year period [2,3]. The concept and advantages of fetal gene therapy were highlighted again in 1995 by several studies on *in vivo* gene delivery in fetal animals [4].

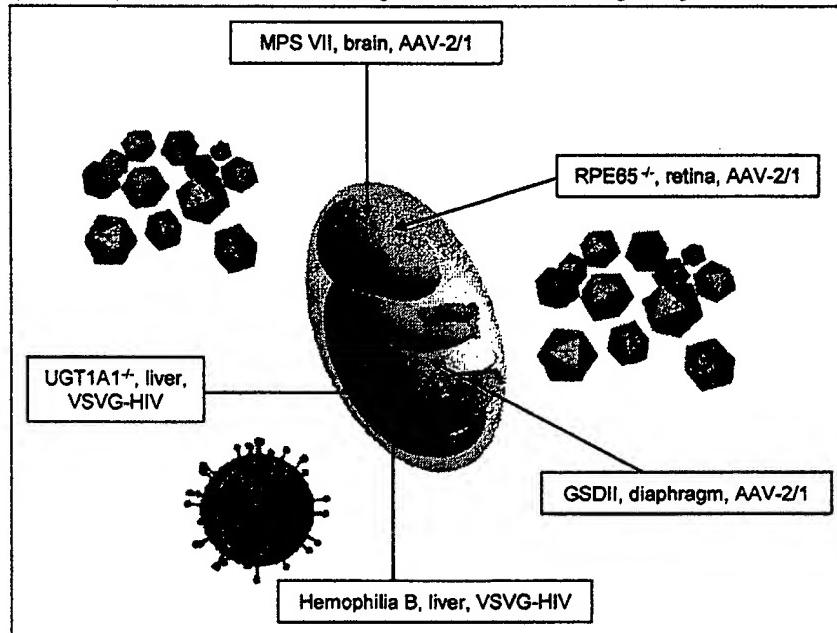
In 1998, the first pre-protocols for human prenatal gene therapy were submitted to the Recombinant DNA advisory Committee. The choice of the two candidate diseases, α -thalassemia and severe combined immunodeficiency caused by adenosine deaminase deficiency (ADA-SCID) were convincingly justified [5]. Four advantages of fetal versus adult gene therapy were hypothesized in this pre-proposal: (i) assuming that stem cells are the principal target, their rapid expansion *in utero* makes them accessible to retroviral vectors and provides a large pool of genetically altered cells; (ii) the small size of the fetus is advantageous when scaling up from therapy in small animals to large animals or humans; (iii) the immunological immaturity of the fetus may result in a reduction in, or even absence of immune/inflammatory reactions against vector and transgenic protein and may permit acquisition of tolerance to the latter; and (iv) the tissue barriers, including the endothelium and epithelium, may be more readily penetrated by vectors in the fetus than in the adult, allowing more extensive vector transduction of organs such as the brain and skin. Therefore, fetal gene therapy could achieve permanent disease correction where adult gene therapy might fail [5].

Preclinical studies

Proof-of-concept

Early attempts to achieve therapeutic gene transfer were reported in 1997 and 1999. One report, describing reversal of lethality in a mouse model of cystic fibrosis after intra-amniotic injection of adenovirus carrying cystic fibrosis transmembrane conductance regulator (CFTR) cDNA, was met with substantial criticism and was not repeatable by other researchers [6]. Another report described transient Factor VIII expression (< 21 days) in haemophilia A mice after injection of adenovirus [7].

Over the past four years, five studies in rodent models have emerged to show proof-of-concept for a postnatal therapeutic effect of *in utero* gene delivery. These are depicted in Figure 1 showing the route of injection, the candidate disease, and the vector implemented. Rucker *et al* used an adeno-associated virus (AAV) serotype 1 vector (AAV-2/1) to deliver human α -glucosidase to the diaphragm of mice deficient in this enzyme, a condition which often results in death from respiratory failure in humans [8••]. Normal contractile function was restored for up to 6 months postpartum. In 2005, Dejneka *et al* used the same AAV serotype to deliver human retinal pigment epithelium 65 (RPE65) to the retinal pigmented epithelium of Rpe65^{-/-} mice and demonstrated restoration of visual

Figure 1. Studies of prolonged postnatal correction of monogenetic disorders following fetal gene transfer to rodents.

Adeno-associated virus (AAV) vectors have been used for the treatment of models of mucopolysaccharidosis VII (MPS VII) [10••], Leber congenital amaurosis [9••] and glycogen storage disease type II (GSDII; Pompe disease) [8••]. HIV-lentivirus vectors have been used to correct models of Factor IX deficiency (hemophilia B) [11••] and bilirubin UDP-glucuronyltransferase deficiency (UGT1A1; Crigler-Najjar type I) [12••]. RPE65 retinal pigment epithelium 65, VSVG vesicular stomatitis virus G.

function [9••]. Karolewski and Wolfe also used AAV-2/1 to treat a mouse model of mucopolysaccharidosis type VII. This vector carrying the human β -glucuronidase cDNA was injected into the ventricle of the fetal brain and resulted in widespread gene expression in the brain and spinal cord and a significant improvement in survival after one year [10••].

All three studies used non-integrating vectors for gene delivery to tissues which undergo minimal cell cycling in adult life. In contrast integration-proficient HIV vectors pseudotyped with vesicular stomatitis virus G (VSVG) were used to correct models of Factor IX deficiency (hemophilia B) in mice, bilirubin uridine diphosphate-(UDP)-glucuronyltransferase (UGT1A1) deficiency (Crigler-Najjar type I) in Gunn rats, and Leber congenital amaurosis in chickens. In the first study, Waddington *et al* injected the vector into the fetal vitelline vessels, which resulted in predominant expression of human Factor IX in the mouse liver (the site of endogenous synthesis) and achieved lifelong correction of the bleeding diathesis [11••]. In the second study, Seppen *et al* achieved intraperitoneal and hepatic delivery of the transgene from direct intrahepatic vector injection. This significantly ameliorated the metabolic defect for at least one year [12••]. In both of the above studies, long-term detection of clusters of transduced cells in liver and intestine, respectively, indicated that organ-specific progenitors had been transduced and were a source of transgene-expressing cells over the rodent's lifetime. In a third study, Williams *et al* describes long-term correction of

a chicken model of Leber congenital amaurosis by lentiviral gene transfer of retinal guanylate cyclase-1 to the neural tube of the embryonic chick [13•]. Early intervention was necessary to pre-empt and prevent photoreceptor cell dysregulation, which precedes degeneration of these cells. These reports of long-term therapeutic benefit of *in utero* gene transfer are supported by several exciting investigations toward fetal gene therapy, which are detailed below.

Consistent with studies demonstrating induction of tolerance to transgenic proteins expressed after fetal gene [14] or stem cell [15] transfer, Sabatino *et al* have demonstrated tolerization to human Factor IX after *in utero* or neonatal administration of AAV [16]. This is supported by Toelen *et al*, who showed that following delivery of a luciferase transgene to rats by various routes of HIV-lentivirus injection, long-term expression of more than 30 weeks was observed [17].

In a model of congenital diaphragmatic hernia induced by nitrofen in rats, Larson and Cohen reported that following delivery of human CFTR to the fetal lungs by intra-amniotic injection of an adenovirus vector, pulmonary hypoplasia (as measured by several parameters of lung pathology including lung volume, internal lung surface area, and number of lung saccules) was ameliorated [18•]. This non-integrating vector was eliminated by dilution from high cell turnover in the respiratory epithelium, after providing the requisite transient expression.

Han *et al* recently reported a study in which therapeutic correction was attempted in an α -thalassemia mouse model by administration of lentivirus at two-thirds gestation [19•]. Although in some animals, expression of α -globin reached up to 20%, this was lost after half a year. This may be symptomatic of a failure to transduce enough erythroid progenitors and/or evidence of transcriptional silencing.

Bilbao *et al* compared AAV-2/1 and AAV-2 after intraperitoneal, intramuscular and intravascular administration *in utero* and established that AAV-2/1 produced the highest transgene expression in the muscle [20]. First-generation and helper-dependent (high capacity/gutless) adenoviral vectors were also compared after intramuscular administration. Increased persistence of vector genomes and reduced toxicity were noted after administration of the helper-dependent adenovirus. These researchers proceeded to show that this vector could be used to deliver full-length murine dystrophin cDNA to fetal *mdx* mice (a mouse model of Duchenne muscular dystrophy). Nine weeks post-delivery, partial restoration of the dystrophin glycoprotein complex in the hind limbs of these mice was demonstrated [21]. Muhle *et al* applied a combination of adenovirus and AAV-2 to a mouse model of Herlitz junctional epidermolysis bullosa [22•]. Both vectors, carrying the cDNA for β 3 chain of laminin-5 protein, were administered into the amniotic fluid. Although laminin-5 was detectable in the basement membranes of skin and mucosae, only a minor increase in survival was observed. Bowers *et al* developed a novel vector combining the high payload capacity of HSV-derived amplicon with the ability of the Sleeping Beauty transposon to mediate integration into the host genome [23]. *In utero* intraventricular injection of this vector resulted in extensive marker gene expression in neuronal precursors for more than 3 months.

Koyama *et al*, Kimura *et al*; and Nakamura *et al* have presented an innovative approach to the gene therapy of pregnancy-related conditions [24-27]. This is based on gene delivery to the extra-embryonic membranes, placenta and uterus, which at least in the first two tissues constitutes bona fide gene transfer to fetal rather than adult tissue. DNA was complexed with Sendai virus hemagglutinating virus of Japan (HVJ) envelope and injected into the uterine cavity but outside the amniotic sac. Expression was observed transiently (for 3 days) in various tissues, including the uterus, placenta, decidua membrane and fetal membrane [24-26]. Transient overexpression of human VEGF was achieved which temporarily reduced the systemic blood pressure of the dam. The researchers suggested that this might be a strategy for treating pre-eclampsia [27].

Compared with *in vivo* vector application, research on *ex vivo* transduction of stem cells before implantation has progressed more slowly and is reviewed more fully in reference [28]. Since this review was published in 2004, Rio *et al* have demonstrated significant engraftment in mice following *in utero* transplantation of hematopoietic stem cells from congenic donors after *ex vivo* transduction with a retroviral vector encoding green fluorescent protein (GFP) [29]. Marker gene expression was detectable in the

peripheral blood cells of these animals as well as in those of secondary recipients transplanted with bone marrow from the *in utero* transplanted mice. Chan *et al* demonstrated engraftment of lentivirus-transduced human fetal mesenchymal stem cells for at least 4 months after injection into *mdx* mice *in utero* [30]. This is particularly interesting, and may be related to the specific immunosuppressive property of mesenchymal stem cells, as Peranteau *et al* have recently demonstrated an immune barrier to allogeneic but not to congenic cell transplants [31]. These researchers used improved procedures via the vitelline vessels to inject adult bone marrow into fetal (14 weeks gestation) recipients. Approximately three quarters of all allogeneic recipients lost engraftment by 1 month whereas all congenic recipients maintained permanent multilineage chimerism. These observations clearly show our underestimation of the complexity of the fetal immune system and indicate that more extensive investigations into the mechanism of tolerance or adverse immune reactions to the delivery of xenoproteins *in utero* are warranted.

Undesirable consequences of gene therapy

As reviewed elsewhere, concerns over the safety of gene therapy are intensified in the context of *in utero* application [32,33]. One of these risks, the inadvertent transduction of the male germline, was assessed in sheep, mice and monkeys. Following intraperitoneal injection of a retroviral vector, PCR on purified sperm cells and immunohistochemistry on testis from the born rams revealed low numbers of transduced germ cells [34]. The researchers estimated a transduction frequency of 1 in 6250 germ cells and noted that this was several orders of magnitude below the calculated frequency of naturally occurring endogenous insertions and below the upper tolerable limit set by the FDA [34]. Analysis in mice after intravascular *in utero* injection of HIV [11•] or AAV [35] vector did not detect any germ cell transduction.

Lee *et al* demonstrated transduction of a subset of oocytes after intraperitoneal injection of VSVG pseudotyped HIV GFP vector into late first trimester macaques [36]. Intrapulmonary or intracardiac routes did not result in germline transduction. The researchers suggested that the ontogeny of the female gonads might predispose them to vector transduction, particularly after intraperitoneal delivery early in gestation [36].

Bedrosian *et al* reported transfer of AAV serotypes (2/1, 2, 5, 7, 8 and 9) and VSVG pseudotyped HIV delivering GFP cDNA to the fetal murine cochlea [37]. Apart from the technical excellence, this study was notable in that the AAV-2/1 vector resulted in optimal expression in the inner and outer hair cells of the cochlea several weeks after injection. However, there was also evidence of mild hearing loss in the lentivirus-injected group. This emphasizes the need for careful consideration of vector-related toxicity in fetal gene transfer protocols.

The high level of cellular proliferation in the fetus, the abundance of growth factors and the transcriptionally active state of genes associated with the regulation of growth and

differentiation may predispose the organism to increased risk of cancer from vectors, which integrate into the host genome. Themis *et al* demonstrated a high incidence of hepatocellular carcinoma following delivery of lacZ or human Factor IX cDNA using an equine infectious anemia virus (EIAV) vector, but not after use of an HIV vector [38]. This suggests that the fetus may be uniquely sensitive to genetic perturbations arising from gene transfer, as EIAV has been used in adult animals with no adverse events reported (reviewed in reference [39]).

Although the majority of studies have resulted in long-term expression and immune tolerance (either apparent or experimentally demonstrable), a few have shown immune or inflammatory reactions following *in utero* gene transfer. Jerebtsova *et al* demonstrated production of low titers of antibody against both the vector and the transgenic protein after delivery of both adenovirus and AAV [40]. The strongest response was elicited after adenovirus administration. As mentioned earlier, Seppen *et al* demonstrated long-term reversal of hyperbilirubinemia in Gunn rats [12••], however, in a later publication these researchers reported the detection of antibodies against UDP-glucuronosyltransferase in both mice and rats [41]. In contrast, injection of control GFP vector did not elicit an anti-GFP response and the researchers concluded that UDP-glucuronosyltransferase may be more immunogenic than GFP.

Large animal models

Fetal gene transfer studies on rodents take advantage of the wide range of disease models, short gestation time and the need for relatively small amounts of vector. However, it is important to translate these studies to larger animal models if the goal of human fetal gene therapy is to be achieved. Larger animal models are valuable since they are more relevant to humans in terms of gestation time, maturation of the fetal immune system, and fetal mass as well as in relation to the vector application technology. Fetal mass is an important factor since this will provide data on how to scale vector dosage. Using the sheep model, David *et al* have adapted and further developed minimally invasive ultrasound-guided transcutaneous techniques, as applied in fetal medicine for gene delivery, to several clinically important fetal organ systems, including the liver, airways, gut and musculature [42,43]. These procedures should permit vector administration to the human fetus (reviewed in reference [44]). For example, David *et al* recently investigated gene transfer to the fetal sheep gut by percutaneous injection using ultrasound guidance [43]. An adenovirus vector carrying the β -galactosidase marker gene was injected directly into the fetal sheep stomach in early second trimester, resulting in expression along the entire gastrointestinal tract in four out of eight individuals. The researchers proposed that this might be useful for *in utero* treatment of the early onset gut pathology of cystic fibrosis. Transduction of fetal sheep airways by ultrasound-guided percutaneous intra-tracheal injection [42] or by direct injection, after exteriorization by laparotomy and hysterotomy [45], has also been achieved recently using first generation adenovirus or VSVG pseudotyped HIV,

respectively. The percutaneous application achieved a high success rate for gene delivery (33 out of 36 fetuses) with good survival (97%) and a low complication rate (6%).

Tarantal *et al* and Jimenez *et al* have used ultrasound after laparotomy to guide administration of VSVG pseudotyped enhanced GFP (eGFP) expressing HIV vectors to the fetal macaque [46,47]. In one study intrahepatic or intraperitoneal injection was administered toward the end of the first trimester. With both routes, expression lasting up to 7 months in various tissues, particularly those of the abdomen, occurred [46]. In a second study GFP expression was observed in the lung, heart and diaphragm after intrapulmonary and intramyocardial injection at mid-gestation [47].

Other animal and delivery models of *in utero* gene transfer technology

Several papers have described other models of *in utero* gene transfer for studies of fetal gene therapy or for broader application as a research tool in areas such as developmental biology. Two reports describe the administration of VSVG pseudotyped HIV vectors to the fetal rabbit after maternal laparotomy. In the first study vector was injected into the amniotic fluid or, after hysterotomy, into the tracheal lumen between 24 and 26 weeks gestation [48]. In the second study, vector was injected into either the fetal peritoneal cavity or directly into the liver between 20 and 22 weeks gestation [49]. In both studies tissues were harvested at 30 weeks gestation and vector was detected by PCR and immunohistochemistry mainly, but not exclusively, in the target organs. Two recent studies have demonstrated the application of ultrasonography for guidance of vector administration to body compartments of the fetal rat after maternal laparotomy and exteriorization of the uterus. One study demonstrated direct injection of adenovirus and EIAV vectors into the lung parenchyma at 15.5 weeks gestation [50] which resulted in gene expression in interstitial cells. The second study demonstrated injection of HIV and EIAV vectors into the amniotic fluid by ultrasound guidance between 8 and 12 weeks gestation, and by direct visualization between 13 and 18 weeks gestation. The extent and depth of eye structures transduced was inversely proportional to the gestational stage of administration and extensive transduction of the lens and cornea was observed up to 6 months after injection [51]. Two reports describe the use of electroporation to deliver genes specifically as research tools. In the first report, the researchers were able to achieve targeting of a sub-population of cells called interneurons after *in utero* gene transfer to the ganglionic eminence [52]. This was achieved by precise positioning of the electroporation electrodes on the fetal cranium and injection of plasmid into the lateral ventricle. The second report, examining spatial and temporal regulation of gene expression, describes the delivery of plasmids incorporating the Cre/loxP tamoxifen-regulated system for controlled expression of marker genes in the brain [53]. Electroporation was performed at 14.5 weeks gestation and the brains were harvested 2 days after birth. Over the past decade, *in vivo* imaging has become an increasingly powerful research technology. Tarantal *et al* have recently used microPET and whole-body *in vivo* bioimaging after fetal

gene transfer of the marker genes thymidine kinase and firefly luciferase to the fetal macaque [54]. Gene expression was observed for up to 21 months, suggesting that these imaging techniques may provide spatial and temporal data for marker gene expression after *in utero* gene delivery in this nonhuman primate model.

Discussion and conclusion

Several alternatives for the management of genetic disease exist, including symptomatic therapies, pre-implantation diagnosis or termination of pregnancy after prenatal diagnosis [33]. For this reason, as discussed elsewhere, the choice of disease for clinical application requires a high level of certainty that benefit will be provided and that the procedure will not cause additional harm.

The submission of first pre-protocols for human fetal gene therapy in 1998 predated the correction of ADA-SCID and X-linked SCID by stem cell gene therapy and the development of leukemia in three of these patients, two of whom were the youngest to be treated. This resulted in the recommendation that a transduction of bone marrow should be avoided in patients below a certain age, as the characteristics of newborn hematopoiesis might predispose them to insertion in the locus implicated in the onset of leukemia [55]. Therefore, although the ideal of fetal gene therapy is to pre-empt and prevent onset of disease, usually by lifelong expression of a transplanted gene to compensate for a single gene defect, the use of integrating vectors for *in vivo* or *ex vivo* gene therapy will certainly depend on further improvement of their safety features.

In the short term two intermediate strategies for possible *in utero* gene therapy are emerging. The first involves the use of '21st Century' non-integrating vectors, including helper-dependent adenovirus, integration-deficient lentivirus, and new adeno-associated virus serotypes. These may be applied to tissues from which the vector genome is slowly lost, such as muscle [80•], neuronal tissue in the central nervous system [10••], and the retina [9••] for longer-term correction of genetic disease. They may also be applied to rapidly dividing tissues, such as the liver, to permit transient correction of genetic diseases which manifest perinatally until a postnatal treatment can be applied; and may also be applied to the fetus, placenta or extraembryonic membranes for treatment of pregnancy-associated diseases such as pre-eclampsia [27] or bronchopulmonary hypoplasia [18•]. The second and archetypal fetal gene therapy strategy [2,3] is *ex vivo* transduction and subsequent re-implantation. This may now exploit the technological advances in maximizing safety and efficacy of *ex vivo* transduction, novel and safer integrating retroviral vectors, and advances in stem cell technology [29,56].

References

- of outstanding interest
 - of special interest
1. Stuhlmann H, Cone R, Mulligan RC, Jaenisch R: Introduction of a selectable gene into different animal tissue by a retrovirus recombinant vector. *Proc Natl Acad Sci USA* (1984) 81(22):7151-7155.
 2. Kantoff PW, Flake AW, Eglitis MA, Scharf S, Bond S, Gilboa E, Erlich H, Harrison MR, Zenjani ED, Anderson WF: *In utero* gene transfer and expression: A sheep transplantation model. *Blood* (1989) 73(4):1068-1073.
 3. Porada CD, Tran N, Eglitis M, Moen RC, Troutman L, Flake AW, Zhao Y, Anderson WF, Zenjani ED: *In utero* gene therapy: Transfer and long-term expression of the bacterial neo' gene in sheep after direct injection of retroviral vectors into preimmune fetuses. *Hum Gene Ther* (1998) 9(11):1571-1585.
 4. Coutelle C, Douar AM, Colledge WH, Froster U: The challenge of fetal gene therapy. *Nat Med* (1995) 1(9):864-866.
 5. Senut MC, Gage FH: Prenatal gene therapy: Can the technical hurdles be overcome? *Mol Med Today* (1999) 5(4):152-156.
 6. Larson JE, Morov SL, Happel L, Sharp JF, Cohen JC: Reversal of cystic fibrosis phenotype in mice by gene therapy *In utero*. *Lancet* (1997) 349(9052):819-820.
 7. Lipshutz GS, Sarker R, Flebbe-Rehwald L, Kazazian H, Gaensler KM: Short-term correction of Factor VIII deficiency in a murine model of hemophilia A after delivery of adenovirus murine Factor VIII *In utero*. *Proc Natl Acad Sci USA* (1998) 95(23):13324-13329.
 8. Rucker M, Fraites TJ Jr, Porvesnik SL, Lewis MA, Zolotukhin I, Cloutier DA, Byrne BJ: Rescue of enzyme deficiency in embryonic diaphragm in a mouse model of metabolic myopathy: Pompe disease. *Development* (2004) 131(12):3007-3019.
 - Restoration of normal contractile function in a mouse model of Pompe disease after intraperitoneal delivery of AAV vector to deliver α -glucosidase.
 9. Dejnaker NS, Surace EM, Aleman TS, Cideciyan AV, Lyubarsky A, Savchenko A, Redmond TM, Tang W, Wei Z, Rex TS, Glover E et al: *In utero* gene therapy rescues vision in a murine model of congenital blindness. *Mol Ther* (2004) 9(2):182-188.
 - Correction of congenital blindness in a mouse model of Leber congenital amaurosis after subretinal injection of AAV vector encoding RPE65.
 10. Karolewski BA, Wolfe JH: Genetic correction of the fetal brain increases the lifespan of mice with the severe multisystemic disease mucopolysaccharidosis type VII. *Mol Ther* (2006) 14(1):14-24.
 - Prevention of CNS storage lesions and early death in a mouse model of mucopolysaccharidosis type VII by intracranial injection of AAV encoding β -glucuronidase.
 11. Waddington SN, Nivsarkar MS, Mistri AR, Buckley SMK, Kemball-Cook G, Mosley KL, Mitrophanous K, Redcliffe P, Holder MV, Brittan M, Georgiadis A et al: Permanent phenotypic correction of haemophilia B in immunocompetent mice by prenatal gene therapy. *Blood* (2004) 104(9):2714-2721.
 - Elimination of the bleeding diathesis in a mouse model of hemophilia B by injection of HIV vector encoding human Factor IX into the vitelline vessels.
 12. Seppen J, van der Rijt R, Locijn N, van Til NP, Lamers WH, Oude Elferink RP: Long-term correction of bilirubin UDP-glucuronyltransferase deficiency in rats by *In utero* lentiviral gene transfer. *Mol Ther* (2003) 8(4):593-599.
 - Prolonged reduction of serum bilirubin in a rat model of Crigler-Najjar type 1 by intraperitoneal injection of HIV vector encoding bilirubin UDP-glucuronyltransferase.
 13. Williams ML, Coleman JE, Haire SE, Aleman TS, Cideciyan AV, Sokal I, Pelczewski K, Jacobson SG, Semple-Rowland SL: Lentiviral expression of retinal guanylate cyclase-1 (RetGC1) restores vision in an avian model of childhood blindness. *PLoS Med* (2006) 3(6):e201.
 - Partial restoration of sight in a chicken model of Leber congenital amaurosis by administration of lentivirus encoding retinal guanylate cyclase-1 to the neural tube *in ovo*.
 14. Waddington SN, Buckley SMK, Nivsarkar M, Jezzard S, Schneider H, Dahse T, Kemball-Cook G, Miah M, Tucker N, Dallman MJ, Themis M, Coutelle C: *In utero* gene transfer of human Factor IX to fetal mice can induce postnatal tolerance of the exogenous clotting factor. *Blood* (2003) 101(4):1359-1366.
 15. Hayashi S, Peranteau WH, Shaaban AF, Flake AW: Complete allogeneic hematopoietic chimerism achieved by a combined strategy of *In utero* hematopoietic stem cell transplantation and postnatal donor lymphocyte infusion. *Blood* (2002) 100(3):804-812.

16. Sabatino DE, Mackenzie TC, Peranteau W, Edmonson S, Campagnoli C, Liu YL, Flake AW, High KA: Persistent expression of hFJX after tolerance induction by *In utero* or neonatal administration of AAV-1-FJX in hemophilia B mice. *Mol Ther* (2007) 15(9):1677-1685.
17. Toelen J, Deroose CM, Gijssbers R, Reumers V, Sbragia LN, Vets S, Chitneni SK, Bormans G, Mortelmans L, Deprest JA, Debeyser Z: Fetal gene transfer with lentiviral vectors: Long-term *In vivo* follow-up evaluation in a rat model. *Am J Obstet Gynecol* (2007) 196(4): 352 e1-6.
18. Larson JE, Cohen JC: Improvement of pulmonary hypoplasia associated with congenital diaphragmatic hernia by *In utero* CFTR gene therapy. *Am J Physiol Lung Cell Mol Physiol* (2006) 291(1):L4-L10.
 - Improvement of pulmonary hypoplasia at birth in a rat model of congenital diaphragmatic hernia by intra-amniotic delivery of adenovirus encoding CFTR.
19. Han XD, Lin C, Chang J, Sadelain M, Kan YW: Fetal gene therapy of α -thalassemia in a mouse model. *Proc Natl Acad Sci USA* (2007) 104(21):9007-9011.
 - Prolonged but eventual loss of expression of human α -globin after delivery of HIV lentivirus to the vitelline vessels in a mouse model of α -thalassemia.
20. Bilbao R, Reay DP, Li J, Xiao X, Clemens PR: Patterns of gene expression from *In utero* delivery of adenoviral-associated vector serotype 1. *Hum Gene Ther* (2005) 16(6):678-684.
21. Bilbao R, Reay DP, Wu E, Zheng H, Biermann V, Kochanek S, Clemens PR: Comparison of high-capacity and first-generation adenoviral vector gene delivery to murine muscle *In utero*. *Gene Ther* (2005) 12(1):39-47.
22. Muhrle C, Neuner A, Park J, Pacho F, Jiang Q-J, Waddington SN, Schneider H: Evaluation of prenatal intra-amniotic LAMB3 gene delivery in a mouse model of Herlitz disease. *Gene Ther* (2006) 13(23):1665-1676.
 - Gene expression but no phenotypic rescue following intra-amniotic injection of AAV and adenovirus vectors encoding the β 3 chain of laminin-5 protein.
23. Bowers WJ, Mastrangelo MA, Howard DF, Southerland HA, Maguire-Zeiss KA, Federoff HJ: Neuronal precursor-restricted transduction via *In utero* CNS gene delivery of a novel bipartite HSV amplicon/transposase hybrid vector. *Mol Ther* (2006) 13(3):580-588.
24. Nakamura H, Kimura T, Ikegami H, Ogita K, Koyama S, Shimoya K, Tsujie T, Koyama M, Kaneda Y, Murata Y: Highly efficient and minimally invasive *In-vivo* gene transfer to the mouse uterus using haemagglutinating virus of Japan (HVJ) envelope vector. *Mol Hum Reprod* (2003) 9(10):603-609.
25. Kimura T, Nakamura H, Koyama S, Ogita K, Tabata C, Tsutsui T, Shimoya K, Koyama M, Kaneda Y, Murata Y: *In vivo* gene transfer into the mouse uterus: A powerful tool for investigating implantation physiology. *J Reprod Immunol* (2005) 67(1-2):13-20.
26. Koyama S, Kimura T, Ogita K, Nakamura H, Tabata C, Md Abu Hadi Noor Ali K, Temme-Asano K, Shimoya K, Tsutsui T, Koyama M, Kaneda Y, Murata Y: Simple and highly efficient method for transient *In vivo* gene transfer to mid-late pregnant mouse uterus. *J Reprod Immunol* (2006) 70(1-2):59-69.
27. Koyama S, Kimura T, Ogita K, Nakamura H, Khan MA, Yoshida S, Watanabe M, Shimoya K, Kaneda Y, Murata Y: Transient local overexpression of human vascular endothelial growth factor (VEGF) in mouse feto-maternal interface during mid-term pregnancy lowers systemic maternal blood pressure. *Horm Metab Res* (2006) 38(10):619-624.
28. Muench MO, Bercena A: Stem cell transplantation in the fetus. *Cancer Control* (2004) 11(2):105-118.
29. Rio P, Martinez-Pelacio J, Ramirez A, Bueren JA, Segovia JC: Efficient engraftment of *In utero* transplanted mice with retrovirally transduced hematopoietic stem cells. *Gene Ther* (2005) 12(4):358-363.
30. Chan J, Waddington SN, O'Donoghue K, Kurata H, Guillot PV, Gotherstrom C, Themis M, Morgan JE, Fisk NM: Widespread distribution and muscle differentiation of human fetal mesenchymal stem cells after intrauterine transplantation in dystrophic mdx mouse. *Stem Cells* (2007) 25(4):875-884.
31. Peranteau WH, Endo M, Adibe OO, Flake AW: Evidence for an immune barrier after *In utero* hematopoietic cell transplantation. *Blood* (2007) 109(3):1331-1333.
32. Waddington SN, Kramer MG, Hernandez-Alcoceba R, Buckley SMK, Themis M, Coutelle C, Prieto J: *In utero* gene therapy: Current challenges and perspectives. *Mol Ther* (2005) 11(5):661-676.
33. Coutelle C, Themis M, Waddington SN, Buckley S, Gregory L, Nivsarkar M, David A, Peebles D, Weisz B, Rodeck C: Fetal gene therapy – first proofs of concept – some adverse effects. *Gene Ther* (2005) 12(22):1601-1607.
34. Porada CD, Park PJ, Tellez J, Ozturk F, Gilimp HA, Almeida-Porada G, Zanjani ED: Male germ-line cells are at risk following direct-injection retroviral-mediated gene transfer *In utero*. *Mol Ther* (2005) 12(4):754-762.
35. Jakob M, Muhrle C, Park J, Weiss S, Waddington S, Schneider H: No evidence for germ-line transmission following prenatal and early postnatal AAV-mediated gene delivery. *J Gene Med* (2005) 7(5):630-637.
36. Lee CC, Jimenez DF, Kohn DB, Tarantal AF: Fetal gene transfer using lentiviral vectors and the potential for germ cell transduction in rhesus monkeys (*Macaca mulatta*). *Hum Gene Ther* (2005) 16(4):417-425.
37. Bedrosian JC, Gratto MA, Brigandt JV, Tang W, Landau J, Bennett J: *In vivo* delivery of recombinant viruses to the fetal murine cochlea: Transduction characteristics and long-term effects on auditory function. *Mol Ther* (2006) 14(3):328-335.
38. Themis M, Waddington SN, Schmitt M, von Kalle C, Wang Y, Al-Allaf F, Gregory LG, Nivsarkar M, Themis M, Holder MV, Buckley SM et al: Oncogenesis following delivery of a non-primate lentiviral gene therapy vector to fetal and neonatal mice. *Mol Ther* (2005) 12(4):763-771.
39. Wong LF, Goodhead L, Prat C, Mitrophanous KA, Kingsman SM, Mazaraki ND: Lentivirus-mediated gene transfer to the central nervous system: Therapeutic and research applications. *Hum Gene Ther* (2006) 17(1):1-9.
40. Jerebissova M, Batshaw ML, Ye X: Humoral immune response to recombinant adenovirus and adeno-associated virus after *In utero* administration of viral vectors in mice. *Pediatr Res* (2002) 52(1):95-104.
41. Seppen J, van Til NP, van der Rijt R, Hirselijk JK, Kunne C, Elferink RP: Immune response to lentiviral bilirubin UDP-glucuronosyl-transferase gene transfer in fetal and neonatal rats. *Gene Ther* (2006) 13(8):672-677.
42. David AL, Weisz B, Gregory L, Themis M, Cook T, Roubilova X, Deprest J, Coutelle C, Rodeck CH, Peebles DM: Ultrasound-guided injection and occlusion of the trachea in fetal sheep. *Ultrasound Obstet Gynecol* (2006) 28(1):82-88.
43. David AL, Peebles DM, Gregory L, Waddington SN, Themis M, Weisz B, Ruthe A, Lawrence L, Cook T, Rodeck CH, Coutelle C: Clinically applicable procedure for gene delivery to fetal gut by ultrasound-guided gastric injection: Toward prenatal prevention of early-onset intestinal diseases. *Hum Gene Ther* (2006) 17(7):767-779.
44. David AL, Themis M, Waddington SN, Gregory L, Buckley SMK, Nivsarkar M, Cook T, Peebles D, Rodeck CH, Coutelle C: The current status and future directions of fetal gene therapy. *Gene Ther Mol Biol* (2003) 7:181-209.
45. Yu ZY, McKay K, van Asperen P, Zheng M, Fleming J, Ginn SL, Kizana E, Latham M, Feneley MP, Kirkland PD, Rowe PB et al: Lentivirus vector-mediated gene transfer to the developing bronchiolar airway epithelium in the fetal lamb. *J Gene Med* (2007) 9(6):429-439.
46. Jimenez DF, Lee CI, O'Shea CE, Kohn DB, Tarantal AF: HIV-1-derived lentiviral vectors and fetal route of administration on transgene biodistribution and expression in rhesus monkeys (*Macaca mulatta*): Safety and efficiency of HIV-1-derived lentiviral vectors for fetal gene delivery. *Mol Ther* (2005) 12(1):87-98.

48. Skarsgard ED, Huang L, Reebye SC, Yeung AY, Jia WW: Lentiviral vector-mediated, *In vivo* gene transfer to the tracheobronchial tree in fetal rabbits. *J Pediatr Surg* (2005) 40(12):1817-1821.
49. Moreno R, Rosal M, Cabero L, Gratacós E, Aran JM: Feasibility of retroviral vector-mediated *In utero* gene transfer to the fetal rabbit. *Fetal Diagn Ther* (2005) 20(6):485-483.
50. Henriques-Coelho T, Gonzaga S, Endo M, Zoltick PW, Davey M, Leite-Moreira AF, Correia-Pinto J, Flake AW: Targeted gene transfer to fetal rat lung interstitium by ultrasound-guided intrapulmonary injection. *Mol Ther* (2007) 15(2):340-347.
51. Endo M, Zoltick PW, Chung DC, Bennett J, Radu A, Muvarak N, Flake AW: Gene transfer to ocular stem cells by early gestational intramniotic injection of lentiviral vector. *Mol Ther* (2007) 15(3):579-587.
52. Borrell V, Yoshimura Y, Callaway EM: Targeted gene delivery to telencephalic inhibitory neurons by directional *In utero* electroporation. *J Neurosci Methods* (2005) 143(2):151-158.
53. Matsuda T, Cepko CL: Controlled expression of transgenes introduced by *In vivo* electroporation. *Proc Natl Acad Sci USA* (2007) 104(3):1027-1032.
54. Tarantal AF, Lee CCI, Jimenez DF, Cherry SR: Fetal gene transfer using lentiviral vectors: *In vivo* detection of gene expression by microPET and optical imaging in fetal and infant monkeys. *Hum Gene Ther* (2006) 17(12):1254-1261.
55. Fischer A, Abina SH, Thrasher A, von Kelle C, Cavazzana-Calvo M: LMO2 and gene therapy for severe combined immunodeficiency. *N Engl J Med* (2004) 350(24):2526-2527.
56. Chen J, O'Donoghue K, de la Fuente J, Roberts IA, Kumar S, Morgan JE, Fisk NM: Human fetal mesenchymal stem cells as vehicles for gene delivery. *Stem Cells* (2005) 23(1):93-102.